LWDB99 AN Cell Death

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(57) Abstract

A method for evaluating the ability of a treatment to adversely affect the growth or viability of a cell is described. A cell having reduced susceptibility to apoptosis is provided, a treatment is administered to this cell, and a determination is made as to whether the treament affects the value of a parameter related to the growth or viability of the cell. Also described are diagnostic and anticancer therapies. Cells and cell lines used in these methods are also provided.

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# ANTICANCER AGENTS AND APOPTOSIS

This application is a continuation-in-part application of pending application Serial No. 08/110,127, filed on August 20, 1993, and entitled ANTICANCER AGENTS AND APOPTOSIS. The entire contents of the parent application are hereby expressly incorporated by reference.

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant Nos. R01CA40602 and 5R27CA17575 awarded by the National Cancer Institute, and Grant Nos. P01-CA42063 and P30-CA14051 awarded by the National Institutes of Health.

## Field of the Invention

This invention relates generally to cancer therapies, e.g, to screens for identifying anticancer agents and to cells for use in such screens.

## Background of the Invention

The identification of effective anticancer agents has long been an objective in medicine. Both radiation-based therapy and chemotherapy have had a significant impact on the treatment of cancer. Major impedients to successful therapy, however, include the failure of some tumor types to respond to either form of treatment, and the appearance of resistant cell populations upon relapse of an originally responsive malignancy. Consequently, the underlying basis of cellular resistance to anticancer agents has been the focus of much experimental study. In general, these investigations have examined how chemotherapeutic agents reach their intracellular targets or the molecular nature of the drug-target interaction. The mechanisms by which anticancer agents cause cell death, however, have thus far remained elusive.

Since ionizing radiation and many chemotherapeutic agents can induce DNA damage or cause disruptions in DNA metabolism, the tumor-specific cytotoxicity of these agents has often been attributed to their genotoxic effect on actively proliferating cells. In many cases, however, the cellular damage caused by therapeutic doses of these agents is not sufficient to explain the observed toxicity.

The process of programmed cell death, or apoptosis, has recently been the subject of various investigations. Apoptosis has been described as physiological cell death, since it is a genetically determined cellular program essential for normal development and maintenance of tissue homeostasis. Cells undergoing apoptosis generally display shrinkage, loss of cell-cell contact, chromatin condensation, and internucleosomal degradation of DNA. Many toxic stimuli have been shown to induce apoptosis, even at doses or concentrations insufficient to cause general metabolic dysfunction.

## Summary of the Invention

One of the major problems in cancer treatment has been the failure of some cancers to respond to chemotherapeutic agents or radiation therapy, as well as the appearance of resistant cell populations upon relapse of an originally responsive cancer to these treatments. Previous screens for anticancer agents have, in general, not focused on the ability of tumor cells to detect cellular damage and activate the apoptotic response, and thus, potentially successful cancer therapies have been missed. The present invention includes a method for identifying new therapies that are effective for many of these previously unresponsive cancers.

According to the invention, a method for evaluating the ability of a treatment to adversely affect the growth or viability of a cell is provided. A cell is provided which has a first condition which reduces the susceptibility of the cell to apoptosis. A treatment is administered to the cell,

and a determination is made as to whether the treatment affects the value of a parameter related to the growth or viability of the cell.

The method provides for a first condition which can include a mutation in a gene that affects the apoptotic pathway, e.g., in a tumor suppressor gene, e.g., the tumor suppressor p53 gene. The mutation can be homozygous or heterozygous. In preferred embodiments, the method also includes a second condition which in a wild type background enhances the susceptibility of the cell to apoptosis. The second condition includes, e.g., an expressed oncogene, e.g., the adenovirus E1A gene or the c-myc gene. In more preferred embodiments, the method also includes a third condition which allows establishment of a permanent cell line when the second condition is present and is an expressed oncogene. The third condition includes, e.g., an expressed oncogene, e.g., T24 H-ras.

In one embodiment of the method of the invention, a second treatment, e.g., a chemotherapeutic agent or radiation is administered to the cell, which in the presence of tumor suppressor gene p53 activity would reduce the growth or viability of the cell. This embodiment allows identification of agents that bypass, restore or replace apoptotic pathway functions.

Variations of this method of this invention include the cell being obtained from a cell culture, e.g., a mouse embryo fibroblast cell culture, or the cell being part of an organism, e.g., an animal, e.g. a transgenic animal, that is wild type or homozygous or heterozygous for a mutation in the apoptotic pathway.

Another aspect of the invention features identifying an agent useful for treating unwanted cell proliferation by providing a cell with a first condition which reduces susceptibility of the cell to apoptosis and a second condition which in a wild type background enhances susceptibility of the cell to apoptosis. An agent is

administered and it is determined if the agent affects the growth rate of the cell, a decrease in the growth rate being correlated with the ability of the agent to treat the unwanted cell proliferation.

Another aspect of the invention features a method for evaluating the ability of a treatment to affect the growth or viability of cells which are wild type for the apoptotic pathway. A first cell is provided which is wild type for the apoptotic pathway, and a second cell is provided which has a mutation in the apoptotic pathway, e.g., in the tumor suppressor p53 gene. A treatment is administered to the first and second cell, and it is determined whether the treatment affects the value of a parameter related to the growth or viability of the first and second cell. The values of the growth or viability parameters between the first and second cell are compared, and if the treatment inhibits the parameter more extensively in the first cell than in the second cell, then the treatment is apoptotic pathway dependent and the treatment is identified as being more effective for cells which are wild type for the apoptotic pathway.

The invention also features a method of utilizing an anticancer drug to alleviate the symptoms of cancer in an organism in which the anticancer drug was originally selected as an anticancer agent by providing a cell which had a first condition which reduced the susceptibility of the cell to apoptosis, administering a first treatment to the cell, and determining whether the first treatment affected the value of a parameter related to the growth or viability of the cell. In one embodiment, a second treatment, e.g., a chemotherapeutic agent or radiation, is utilized in combination with the first treatment to alleviate the symptoms of cancer in the organism.

Yet another aspect of the invention features a method for treating an organism with cancer in which the organism's cancer cells are tested for the presence or absence of a

tumor suppressor gene p53 mutation. If a p53 mutation is absent, then a therapeutically effective amount of a treatment is administered, the treatment being a p53-dependent treatment, or a combination of a p53-dependent and a p53-independent treatment. If a p53 mutation is present, then a therapeutically effective amount of a treatment is administered, the treatment being a p53-independent treatment, or a sufficiently high level of a normally p53-dependent treatment so as to overcome the p53-dependent property of the p53-dependent treatment.

And yet another aspect of the invention includes a method of treating a cell which is tumorigenic by administering a therapeutically effective amount of tumor suppressor gene p53 protein or DNA in an amount sufficient to give expression of a therapeutically effective amount of p53 protein. Preferably, the cell is deficient in tumor suppressor gene p53 function. In one embodiment, the cell is part of an organism. In another embodiment, the cell is obtained from a tumorigenic cell culture, and additionally, a therapeutically effective amount of treatment with a chemotherapeutic agent or radiation is administered.

In addition, mouse embryonic fibroblast cells that are either homozygous or heterozygous for a tumor suppressor gene p53 mutation and have an adenovirus E1A gene and a T24 H-ras gene are provided. Mouse embryonic fibroblast cell lines that are either homozygous or heterozygous for a tumor suppressor gene p53 mutation and have an adenovirus E1A gene and T24 H-ras gene are also provided.

It is an object of the invention to evaluate the ability of a treatment to adversely affect the growth or viability of a cell.

It is another object of the invention to identify anticancer agents.

It is yet another object of the invention to identify anticancer agents that are effective for cancers that are not responsive to currently available chemotherapeutic agents or radiation therapy.

It is yet another object of the invention to identify agents which can retard the growth of cultured cells, e.g., transformed cultured cells.

It is yet another object of the invention to identify agents which can retard the growth of tumor cells that are part of an organism.

It is yet another object of the invention to provide a cheap, easy and fast method for screening large numbers of putative anticancer agents.

It is yet another object of the invention to identify anticancer agents that are not cytotoxic to nonmalignant cells.

It is yet another object of the invention to identify anticancer agents that are apoptotic pathway-independent.

It is yet another object of the invention to identify anticancer agents that are apoptotic pathway-dependent.

It is yet another object of the invention to identify anticancer agents that bypass, restore or replace an apoptotic pathway gene function.

It is yet another object of the invention to provide an in vitro test system which permits evaluations and comparisons between apoptotic pathway-dependent and apoptotic pathway-independent anticancer agents.

It is yet another object of the invention to provide an in vivo test system which permits evaluations and comparisons between apoptotic pathway-dependent and apoptotic pathway-independent anticancer agents.

It is yet another object of the invention to identify the genetic requirements for drug cytotoxicity.

It is yet another object of the invention to screen an organism's cells for the presence or absence of mutations in the apoptotic pathway and then, depending upon the outcome of the screen, administer an effective anticancer treatment.

It is yet another object of the invention to use an anticancer agent identified in an in vitro test system as apoptotic pathway-independent or apoptotic pathway-dependent, to alleviate the symptoms of cancer in an organism.

Still another object of the invention is to treat an organism with a tumor by administering tumor suppressor gene p53 protein or DNA.

The above and other features, objects and advantages of the present invention will be better understood by a reading of the following specification in conjunction with the drawings.

## Brief Description of the Drawings

FIG. 1 depicts dose response curves for the viability of wild type and p53-deficient cells after irradiation.

FIG. 2 depicts dose response curves for the viability of wild type and p53-deficient cells after treatment with chemotherapeutic agents.

FIG. 3 depicts dose response curves for the viability of cells treated with sodium azide.

FIG. 4 depicts a 24-well plate for assaying putative anticancer agents at different concentrations in wild type and p53-deficient cells.

FIG. 5 depicts hypothetical dose response curves for the viability of wild type and p53-deficient cells after treatment with putative anticancer agents.

FIG. 6 depicts hypothetical dose response curves for plating efficiency of wild type and p53-deficient cells after treatment with putative anticancer agents.

FIG. 7 depicts hypothetical dose response curves for plating efficiency of wild type and p53-deficient cells after treatment with p53-dependent drugs and putative anticancer agents.

FIG. 8 depicts in vivo tumor response as a function of time to radiation or chemotherapy in mice injected with wild type or p53-deficient cells.

FIG. 9 depicts acquired resistance to radiation or chemotherapy of tumors derived from p53-expressing cells.

## Detailed Description

This invention provides a method for evaluating the ability of a treatment to adversely affect the growth or viability of a cell. A cell is provided which has a first condition which reduces the susceptibility of the cell to apoptosis. In a preferred embodiment, the cell also has a second condition which when present in a wild type background enhances the susceptibility of the cell to apoptosis. And, in a more preferred embodiment, when the second condition is present, the cell also has a third condition which allows establishment of a permanent cell line when the second condition is an expressed oncogene. A treatment is administered to the cell, and the usefulness of the treatment as an anticancer agent is evaluated, e.g., by determining the effect of the treatment on the value of a parameter that is related to the growth or viability of the cell.

The term cell is meant to include a cell or a group of cells. The cell can be a human or a nonhuman cell. The cell may be obtained from a culture including a primary cell culture, an early passage cell culture, and an immortalized cell culture. Preferably, the cell is obtained from an early passage cell culture. An early passage cell culture results from subculturing a primary cell culture. To avoid possible selection for mutations prior to employing a screen of this invention, cells from an early passage cell culture are preferably obtained prior to the fourth subculturing. Kidney cells and mouse embryo fibroblasts, as well as many other living cells, can be used in methods of the invention. Preferably, the cell is a mouse embryo fibroblast.

The cell of the invention may also be part of an organism. The term organism is meant to include human as well as nonhuman animals. Animals include mammals, birds, reptiles, amphibians, insects and fish. Preferred animals are mammals and preferred mammals are humans or nonhuman mammals, e.g., monkeys, pigs, dogs, cats, sheep, goats, cows, horses, rabbits and rodents. An organism includes transgenic

nonhuman animals. The term transgenic animal is meant to include an animal that gains new genetic information from the introduction of foreign DNA or a lesion, e.g., an in vitro induced mutation, e.g., a deletion or other chromosomal rearrangement, into the DNA of its cells. The animal may include a transgene in all of its cells including germ line cells, or in only some of its cells. Preferably, the transgenic animal is a mouse.

The term condition means a state of the cell. Such a condition includes, e.g., the state of the genome, the level of a gene product, and the introduction of any type of agent into or onto the cell. The state of the genome includes, e.g., a mutation in a gene and the addition of an exogenous gene. An agent includes, e.g., a molecule and radiation.

By apoptosis is meant programmed cell death. Such programmed cell death is at least in part a genetically controlled program essential for normal development and maintenance of tissue homeostasis. Cells undergoing apoptosis generally display one or more of shrinkage, loss of cell-cell contact, chromatin condensation and internucleosomal degradation of DNA. Many toxic stimuli induce apoptosis, even at doses or concentrations insufficient to cause general metabolic dysfunction. In addition, the expression of oncogenes may sensitize cells to apoptosis.

A condition which can reduce the susceptibility of a cell to apoptosis can include, e.g., a mutation in a gene that affects an apoptotic pathway. The term mutation is meant to include lesions which increase or decrease the level of expression of a gene affected by the mutation. The mutation can be homozygous or heterozygous. Genes that affect the apoptotic pathway include tumor suppressor genes, preferably the p53 gene, (Levine et al. Nature 351: 453-456, 1991), and regulators of tumor suppressor genes, e.g., the mdm2 gene which is a regulator for the p53 gene. Thus, absence of the p53 gene product reduces the susceptibility of

a cell to apoptosis. Another condition that can reduce the susceptibility of a cell to apoptosis includes the presence of certain selected oncogenes, the expression of which inhibits apoptosis. Preferred oncogenes are bcl2, HPV-E6 and adenovirus E1B. Yet another condition that can reduce susceptibility is the presence of a nonwild type level of expression of a gene which affects the apoptotic pathway.

In preferred embodiments, the cell also has a second condition which in a wild type background enhances the susceptibility of a cell to apoptosis. In preferred embodiments the second condition at least partially transforms the cell of the invention. By transformed it is meant the failure to observe the normal constraints of growth. Transformed cells usually are characterized by one or more of the following: growth in a much less restricted manner than a normal cell, including generally dividing far more frequently; generally not needing a solid surface to which to attach; generally having reduced serum-dependence; generally piling up into a thick mass of cells instead of being restricted to a thin layer on the surface; and generally inducing tumors when injected into an appropriate organism. The second condition can, e.g,. include certain expressed oncogenes other than those referred to in the previous paragraph. The oncogene includes the adenovirus E1A gene and the c-myc gene. In the absence of the p53 gene product, programmed cell death of an E1A transformed cell is greatly reduced.

Cells which have the above-described second condition may also have a third condition which allows establishment of a permanent cell line when the second condition is an expressed oncogene. The third condition allows the cell to form a tumorigenic cell line. The third condition can include, e.g., an expressed oncogene, preferably T24 H-ras.

Thus, in the preferred embodiment, the cell has (i) a mutation in the tumor suppressor gene p53 which reduces the susceptibility of the cell to apoptosis, in that wild type

p53 is normally required for programmed cell death to occur, (ii) an expressed adenovirus E1A oncogene which in a wild type background enhances the susceptibility of the cell to apoptosis, and therefore results in cell death, but which in the absence of p53 gene product transforms the cell to a malignant state, and (iii) an expressed T24 H-ras oncogene which allows establishment of a permanent tumorigenic cell line when the expressing E1A oncogene is present.

Such a malignant cell is not killed by the cell's programmed cell death pathway (apoptosis) because of the absence of the apoptotic pathway p53 gene product. Treatments which are identified as being able to result in death of such cells are anticancer agents of great importance, in that p53 mutations are observed in many different types of cancers that are resistant or that become resistant to known chemotherapeutic drugs and ionizing radiation (Stretch et al., Cancer Res. 51: 5976-5979; Chiba et al., Oncogene 5: 1603-1610, 1990; Sidransky et al., Nature 355: 846-847, 1992; Felix et al., J. Clin. Invest. 89: 640-647, 1992; Yeargin et al., J. Clin. Invest. 91: 211-217, 1993.

The term treatment is meant to include an anticancer agent or putative anticancer agent that is administered. An anticancer agent can be, e.g., radiation or a molecule, e.g., a chemotherapeutic drug. Anticancer agents include, e.g., agents which kill cells, or prevent or retard growth and/or reproduction of cells. Preferably, the agents have a greater effect on actively dividing cells as compared to nondividing cells. Administration of the treatment includes introduction into, onto, in the vicinity of, e.g., into a medium in which the cell is suspended, or distal to, the cell. Depending upon the type of treatment, and the state of the cell, e.g., whether it is in a cell culture or part of an animal, the appropriate method for administration is chosen. The time, temperature, concentration and dose of the treatment depends

upon the specific cells being used and the specific treatment being tested. Preferably, a range of parameters are used for any putative treatment that is being assayed.

Subsequent to administration of the treatment, a determination is made as to whether the treatment affects the value of a parameter related to the growth or viability of the cell. The growth or viability of the cell can be determined in a variety of ways, including a plate viability assay, a colony regression assay, a plating assay, and a progression or regression assay of tumors in animals.

Evaluating the ability of a treatment to affect a property of a cell includes, e.g., screening or testing the cell for the presence of the property. In a preferred embodiment, the invention excludes doing this evaluating on a cell or organism which is concurrently being treated for a disease characterized by unwanted cell proliferation, e.g. cancer.

In methods of the invention, anticancer agents are identified in cells which are defective in the p53 apoptotic pathway because of some mutational event or other reason, as well as in p53 apoptotic pathway wild type cells. Thus, these assays identify anticancer agents that are either apoptotic pathway-dependent or apoptotic pathway-independent. By apoptotic pathway-dependent, it is meant an anticancer agent that requires a functioning p53 gene in order to be an effective anticancer agent. By apoptotic pathway-independent, it is meant an anticancer agent that does not require a functioning p53 gene in order to be an effective anticancer agent. Example 11 describes in detail a preferred embodiment for identifying such anticancer agents. In addition, methods of this invention can also indicate whether the anticancer treatment is cytotoxic to normal cells in addition to tumorigenic cells.

In one embodiment of the invention, a method for utilizing an anticancer drug which was originally selected as

an anticancer agent by the methods of this invention described above, to alleviate the symptoms of cancer in an organism, is also provided.

One of the major problems with current cancer therapies is the appearance of resistant cell populations. This invention includes the administration of apoptotic pathway-dependent and apoptotic pathway-independent treatments, in combination, in order to minimize the problem of drug resistance. This problem is reduced by combination therapy because it is highly unlikely that the same cell would undergo mutations in both of these separate pathways. Therapeutic agents of the invention can be administered in any appropriate mode, e.g., orally, intravenously, subcutaneously, intraperitoneally, topically, combined with a liposome, in a time release formulation, transgenically, or by irradiation.

In a variation, the method provides for evaluating the ability of a treatment to bypass or restore or replace the function of a mutated or inactivated gene in the apoptotic pathway, e.g., a mutation in the tumor suppressor p53 gene. The method is similar to that described above, except that a treatment, e.g., a chemotherapeutic agent or radiation, that, preferably is known to kill the cell in the presence of tumor suppressor gene p53 activity, is administered in addition to administering the agent that is to be evaluated. In the absence of a functioning apoptotic pathway gene, the cell is not killed by the known chemotherapeutic agent or radiation. If the test agent restores or bypasses or replaces the function of the mutated or inactivated gene in the apoptotic pathway, the cell then becomes subject to programmed cell death.

This invention further provides a method for treating an organism, cell culture or cell, characterized by unwanted cell proliferation. Unwanted cell proliferation includes, e.g., cancer. By treating unwanted cell proliferation, e.g., cancer, it is meant alleviating the adverse effects of

tumorigenic cells, and/or eliminating, killing or shrinking the tumorigenic cells. In this method, the proliferating, e.g., cancer, cells are tested for the presence or absence of a tumor suppressor gene p53 mutation. If a p53 mutation is absent in the proliferating cell, then a therapeutically effective amount of a treatment is administered. Such treatment includes a p53-dependent treatment, or a combination of a p53-dependent and a p53-independent treatment. By p53-dependent it is meant that the treatment requires tumor suppressor gene p53 product in order to be effective as a treatment. A p53-independent treatment does not require tumor suppressor gene p53 product. If a p53 mutation is present in the proliferating cell, then the treatment includes administration of a p53-independent treatment, or a sufficiently high level of a normally p53-dependent treatment so as to overcome the p53-dependent property of the p53-dependent treatment.

This invention also includes a method of treating a tumorigenic cell culture, or an organism with a tumor, preferably, the cells of which are deficient in the expression of a gene in the apoptotic pathway, by administering a therapeutically effective amount of the apoptotic pathway gene protein or DNA in an amount sufficient to give expression of a therapeutically effective amount of the apoptotic pathway protein. In a preferred embodiment, the mutation is in the p53 gene. In preferred embodiments, p53 protein or DNA is administered. By therapeutically effective amount it is meant an amount sufficient to give a desired result when presented to a cell as, e.g., a cell in a cell culture or a cell in a living organism. Desired results include retardation of unwanted cell growth and/or reproduction, destruction of cells and loss of viability or unwanted characteristics. The method also may include administering a second treatment, for example a chemotherapeutic agent or radiation, in addition to the p53

protein or DNA. In the presence of the p53 protein or DNA, p53 deficient cells can become responsive to this sequence treatment.

This invention also includes mouse embryonic fibroblast cells which are wild type for the tumor suppressor gene p53 or are either homozygous or heterozygous for a tumor suppressor gene p53 mutation, and preferably also have an adenovirus E1A gene, and most preferably also have a T24 H-ras gene. This invention further includes mouse embryonic fibroblast cell lines comprising cells that are wild type for the tumor suppressor gene p53 or are either homozygous or heterozygous for a tumor suppressor gene p53 lesion and preferably also have an adenovirus E1A gene, and most preferably also have a T24 H-ras gene.

Deposit of MEF cells which $p53^{-/-}$ and have an adenovirus E1A gene and a T24 H-ras gene (strain 1AR.A9) has been made on August 19, 1993, with the American Type Tissue Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, and has been assigned deposit number CRL 11437. Deposit of MEF cells which are $p53^{+/+}$ and have an adenovirus E1A gene and a T24 H-ras gene (strain 1AR.C8) has been made on August 19, 1993, with the American Type Tissue Collection, 12301 Parklawn Drive, Rockville, MD 20852, and has been assigned deposit number CRL 11438.

## EXAMPLES

Example 1 - Preparation of Embryonic Fibroblasts

$p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were obtained from 12-15 day embryos derived from crosses between mice in which one p53 allele had been disrupted by gene targeting. (Livingstone et al., Cell 70: 923-935, 1992). Mice with these genotypes are available from GenPharm International, Mountain View, California (TSG-p53 © transgeneic mouse, item numbers P53101, P53102), P53201, P53202, P53301, P53302), and Jackson Laboratory, Bar Harbor, Maine (catalog # JR2080). The lack of p53 expression

in homozygous mutant fibroblasts was confirmed. (Livingstone et al., Cell 70: 923-935, 1992). Embryo genotypes were determined using a polymerase chain reaction assay that distinguishes between the wild type and mutant p53 alleles (Livingstone et al., Cell 70: 923-935, 1992).

To isolate fibroblasts, the heads of the embryo were removed and the "red" organs were dissected away (heart and liver). These embryos were minced and incubated in trypsin for 0.5-1 hour at 37°C. By occasionally passaging cells through a pipette, a single cell suspension is obtained. Cells were transferred to tissue culture plates or flasks at subconfluent density (2-4 x $10^6$ cells/150 $cm^2$ flask) into Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum (FBS). One embryo generated 2-4 150 $cm^2$ flasks. These cultures are designated "primary" or "first passage" cultures. Cells were grown in DME in 10% FBS in an incubator containing 5% $CO_2$ at 37°C, until they reached confluence, after which they were plated out for gene transfer or frozen for subsequent use.

Example 2 - Gene Transfer

Upon subculturing "primary" cultures, cells are referred to as "early passage" cultures. To avoid possible selection for genetic alterations prior to gene transfer, MEFs from Example 1 were used prior to the fourth subculturing. The adenovirus E1A gene (Shenk and Flint, Adv. Cancer Res. 57: 47-85, 1991) was introduced into these cells using gene transfer vectors (plasmids or retroviruses) that also express a suitable drug resistance marker. It was important that the drug resistance gene was on the same vector as E1A in order to insure that drug-resistant colonies had a high probability of co-expressing the gene of interest. In addition, as described below, cells were constructed which also had either an activated ras oncogene, T24 H-ras (Franza et al., Cell 44: 409-418, 1986), or an adenovirus E1B gene, which was co-introduced with E1A. Stable lines were selected in the appropriate concentrations of drug.

Specifically, E1A was introduced into MEFs using p1AHygro, a plasmid derived by insertion of an Ad5 genomic E1A fragment (nucleotides 1-1834) (Lowe and Ruley, Genes Dev. 7: 535-545, 1993) into pY3 (a plasmid expressing hygromycin phosphotransferase) (Blochinger and Diggelmann, Mol. Cell. Biol. 4: 2929-2931, 1984). MEFs were seeded at $10^6$ cells/100mm dish and allowed to adhere overnight. Exogenous DNA was introduced by calcium phosphate co-precipitation (Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, N.Y., 1989) using 1 µg p1AHygro to generate E1A containing cells. To generate E1A and T24 H-ras containing cells, 1µg p1AHygro plus a 10-fold molar excess of pT24 neo was used. pT24neo encodes an oncogenically activated human ras allele (T24 H-ras) expressed by its endogenous promoter. (Franza et al., Cell 44: 409-418, 1986). To generate E1A and E1B containing cells, 1 µg p1AHygro plus a 10-fold molar excess of p5XX was used. P5XX encodes the E1B gene. (Lowe and Ruley, Genes Dev. 7: 535-545, 1993). For each precipitation, the total mass of DNA was adjusted to 20 µg using a control plasmid, pBluescript, obtained from Stratagene of LaJolla, California. Precipitates were incubated with cells for 4 hours in the presence of 100 µM chloroquine, washed, and incubated for 24 hours in normal growth media. Subsequently, cultures were split into 3-5 150 mm plates and put on media containing either 100 µg/ml (for $p53^{-/-}$ MEFs) or 20 µg/ml (for $p53^{+/+}$ and $p53^{+/-}$ MEFs) Hygromycin B (obtained from Sigma Chemical Co. of St. Louis, Missouri). Fresh selection media was added every 3-4 days. After 2-3 weeks, colonies were either analyzed directly or expanded into permanent cell lines. All experiments were performed in hygromycin-free media.

This procedure results in oncogenes which enhance susceptibility to apoptosis in a wild type background being present in the cells at a level of at least about one copy per cell.

Alternatively, other oncogenes, e.g., c-myc, (Evan et al., Cell 61: 759-767), can be introduced into the cells in place of the E1A gene.

Example 3 - Colony Regression Assays

In this example, known anticancer agents are tested to show that certain colonies regress in size when exposed to such agents.

p1AHygro, which encodes the adenovirus-5 E1A gene and hygromycin phosphotransferase, was transfected into $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs obtained from Example 1, by calcium phosphate co-precipitation. Thus, colonies arising in hygromycin B had a high probability of co-expressing E1A. E1A-expressing colonies have a distinct morphology that distinguishes them from normal or hygromycin-resistant MEFs, and have been shown to express E1A by immunofluorescent staining. Approximately 3 weeks after transfection, cultures were transferred to normal growth medium (without Hygromycin B), and E1A-expressing colonies were marked and treated with 5 grays (Gy) ionizing radiation using a Gammacell 40 irradiator (produced by Atomic Energy of Canada Limited, Commericial Products, of Ottawa, Canada), equipped with a $^{137}Cs$ source. Alternatively, colonies were incubated in the presence of 1 μM 5-fluorouracil (Sigma), 0.2 μM etoposide (Sigma), or 0.2 μg/ml adriamycin (Sigma). Selected colonies were photographed at various times thereafter. All colonies were scored for significant regression 72 hours after treatment, except for colonies incubated in adriamycin, which were scored for regression after 24 hours.

Example 4 - Dose-Response Assays

For irradiation experiments, exponentially growing cells from Example 2 were detached from plates and adjusted to $10^6$ cells/ml. Samples were irradiated for different times, and 1 ml of each cell suspension was added to 100 mm dishes

containing normal growth medium. Cell viability was assessed 36 hours after irradiation by pooling adherent and nonadherent cells and measuring uptake of fluoresceine isothiocyanate (FITC) by FACS analysis (Shi et al., J. Immunol. 144: 3326-3333, 1990; Lowe et al., Genes Dev. 7: 535-545, 1993). At least $10^5$ cells were measured for each determination. Cells used in chemical cytotoxicity experiments were plated at 1-2 x $10^6$ cells/100mm dish, allowed to adhere, and incubated with various concentrations of 5-fluorouracil, etoposide, adriamycin, or sodium azide (Sigma). Cell viability was determined 24 hours following treatment.

In order to confirm that cell death occurred by apoptosis, cells were analyzed for fragmented DNA. 24 hours after treatment, adherent and nonadherent cells were pooled and counted. Low molecular weight DNA was isolated from 2 x $10^6$ cells and visualized following electrophoresis on 1% agarose gels and staining with ethidium bromide. Example 8 below sets forth the results obtained when following the above protocol.

Example 5 - Cell Cycle Analysis

Cellular proliferation was assessed by DNA content and incorporation of 5-bromo-2'-deoxyuridine (BrdU) using multiparameter flow cytometry analysis. Cells from Example 2 were plated in growth medium at 1-2 x $10^6$ cells/100mm dish, allowed to adhere, and exposed to 5 Gy ionizing radiation. 14 hours after treatment, BrdU (obtained from Amersham Life Sciences of Arlington Heights, Illinois) was added and the cultures were incubated at 37°C for an additional 4 hours. Cultures were washed twice with PBS to remove dead cells, and the adherent cells were collected after treatment with trypsin. The cells were washed in PBS (phosphate buffered saline) and fixed in 70% ethanol for 30 minutes at -20°C. Samples were prepared and analyzed for multiparameter FACS analysis (van Erp et al., Cytometry 9: 627-630, 1988). The

percentage of cells in each phase of the cell cycle (at the end of the BrdU labeling period) was estimated using the MODFIT program (obtained from Verity Software House of Topsham, Maine). The co-efficient of variance for each fit was usually less than 5%. The percentage of cells incorporating BrdU during the 4 hour pulse was estimated from the log red fluorescence and log green fluorescence plot using the disp2D program (obtained from Becton Dickinson and Co. of San Jose, California).

Example 10 below sets forth the results obtained when following the above protocol.

Example 6 - E1A-expressing Cells Rapidly Undergo p53-dependent Cell Death Following Exposure To Ionizing Radiation

Due to the difficulty in establishing cell lines that continuously express E1A, cell viability was analyzed using unexpanded colonies derived following introduction of E1A into early passage mouse embryo fibroblasts (MEFs). As described in Example 2, $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs were transfected with p1AHygro, a plasmid co-expressing both adenovirus-5 E1A and the hygromycin phosphotransferase gene, and E1A-expressing colonies were selected in hygromycin B, as described in Example 2, except that pT24neo was not introduced. In a separate series of experiments, a plasmid encoding the adenovirus E1B gene (which counteracts the effects of p53 (Debbas and White, Genes Dev. 7: 546-554, 1993) was co-introduced with p1AHygro, as described in Example 2. After approximately 3 weeks in hygromycin-containing medium, E1A-expressing colonies were marked and inspected for regression after exposure to 5 grays (Gy) ionizing radiation.

The viability of colonies exposed to 5 Gy ionizing radiation is summarized in Table 1.

TABLE 1

COLONY REGRESSION FOLLOWING TREATMENT WITH IRRADIATION

| Treatment | Exogenous Genes | p53 Genotype | Colony Viability Regressing | Resistant | % Resistant |
|---|---|---|---|---|---|
| Radiation | E1A | (+/+) | 5 | 1 | 17 |
| | E1A | (+/-) | 25 | 0 | 0 |
| | E1A | (-/-) | 1 | 24 | 96 |
| | E1A + E1B | (+/+) | 4 | 9 | 69 |
| | E1A + E1B | (+/-) | 9 | 16 | 64 |
| | E1A + E1B | (-/-) | ND | ND | ND |

While irradiation of untransfected fibroblasts had no effect on cellular viability, virtually all $p53^{+/+}$ and $p53^{+/-}$ colonies expressing E1A rapidly degenerated. By contrast, $p53^{-/-}$ colonies expressing E1A were resistant to irradiation, indicating that cell death required p53 function. Although not as effective as the absence of p53, co-expression of E1B protected E1A-expressing $p53^{+/+}$ cells from death following irradiation.

Example 7 - Genotoxic Compounds Used in Cancer Chemotherapy Induce p53-dependent Cell Death in Cells Expressing E1A in the Presence of the p53 Gene Product

A variety of genotoxic compounds used as chemotherapeutic agents were tested for their ability to induce p53-dependent cell death in E1A-expressing cells using the colony regression assay described in Example 3. 5-fluorouracil (anti-metabolite), etoposide (topoisomerase II inhibitor) and adriamycin (intercalating agent) were chosen because they have different intercellular targets (Chabner and Myers, in Cancer Principles and Practice of Oncology, DeVita et al., eds., J.B. Lippencott Co., Philadelphia, pp. 349-395, 1989).

plAHygro was transfected into $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs obtained from Example 1 and colonies were selected in hygromycin B. Approximately 3 weeks after transfection, the colonies were marked and the liquid media

was replaced with liquid media containing either 1μM 5-fluorouracil, 0.2 μM etoposide, or 0.2 μg/ml adriamycin in the absence of Hygromycin B. Colonies were inspected by microscopy for significant regression and cell death 72 hours after initiating treatment, except for colonies incubated in adriamycin which were scored after 24 hours.

As observed following irradiation, $p53^{+/+}$ and $p53^{+/-}$ colonies that expressed E1A displayed a remarkable sensitivity to each of these agents: the majority of colonies completely regressed within 72 hours of treatment, as summarized in Table 2.

TABLE 2

COLONY REGRESSION FOLLOWING TREATMENT WITH CHEMOTHERAPEUTIC AGENTS

| Treatment | Exogenous Genes | p53 Genotype | Colony Viability (72 h) Regressing | Resistant | % Resistant |
|---|---|---|---|---|---|
| 5-fluorouracil | E1A | (+/+) | 5 | 0 | 0 |
| | E1A | (+/-) | 23 | 2 | 8 |
| | E1A | (-/-) | 1 | 26 | 96 |
| Etoposide | E1A | (+/+) | 5 | 1 | 17 |
| | E1A | (+/-) | 20 | 5 | 20 |
| | E1A | (-/-) | 0 | 25 | 100 |
| Adriamycin | E1A | (+/+) | 3 | 0 | 0 |
| | E1A | (+/-) | 22 | 3 | 12 |
| | E1A | (-/-) | 0 | 25 | 100 |

Again, cell death required p53 function, since $p53^{-/-}$ cells were resistant to all treatments, although p53-deficient colonies did show some degree of regression after several days in adriamycin. The vast majority of E1A-expressing colonies derived from $p53^{+/-}$ MEFs degenerated completely following treatment with various genotoxic compounds; however, a small number retained viability even after 6 days. By using a polymerase chain

reaction assay that distinguishes between mutant and wild type p53 alleles, three out of four resistant colonies were shown to have lost the wild type p53 allele, and therefore had become deficient for p53. These data indicate that E1A increased cellular sensitivity to a number of chemotherapeutic agents and that ensuing cell death was dependent on a functional p53 gene.

Example 8 - Anticancer Agents Trigger p53-dependent Apoptosis in Cells Co-expressing E1A And T24 H-ras In The Presence Of The p53 Gene Product

The effects of various anticancer agents on permanent cell lines expressing E1A were examined. Although primary cells expressing E1A and endogenous p53 could not be expanded, $p53^{+/+}$ cells transformed by E1A and ras oncogenes (T24 H-ras), as described in Example 2, could be readily established. Cells expressing T24 H-ras and E1A remained susceptible to apoptosis upon serum withdrawal.

The viability of wild type and p53-deficient cells after irradiation was determined, as shown in FIG. 1. The viability of cells expressing (FIG. 1, panel A) or lacking (FIG. 1, panel B) endogenous p53 was measured by FITC uptake and FACS analysis 36 hours after treatment with the indicated dose of ionizing radiation. Each point represent the average and standard deviation obtained from at least 3 independent clones. All values were normalized to the relative viability of the corresponding untreated controls from the same experiment (generally greater than 90%). Open circles represent untransfected MEFs; closed circles, $p53^{-/-}$ clones expressing E1A; squares, clones co-expressing E1A and T24 H-ras; triangles, cells expressing E1A and E1B.

Like cells expressing E1A alone, exposure of $p53^{+/+}$ cells co-expressing E1A and T24 H-ras to ionizing radiation caused a dose-dependent decrease in viability, with significant death occurring at doses as low as 1 Gy.

Radiation treatment had a minimal effect on all $p53^{-/-}$ lines and untransfected fibroblasts, and most cells retained viability after treatment with 20 Gy.

In addition, it has been determined that $p53^{-/-}$ cells co-expressing E1A and T24 H-ras irradiated with 5 Gy displayed no significant loss of growth or tumorigenic potential. $p53^{+/-}$ cells co-expressing E1A and T24 H-ras died following irradiation, but to a considerably lesser extent than wild type cells. Ionizing radiation, however, had no effect on the viability of either $p53^{+/+}$ or $p53^{-/-}$ untransfected MEFs. E1B, which inhibits E1A-associated apoptosis (Rao et al., Proc. Nat'l Acad. Sci. USA 89: 7742-7746, 1992; Lowe and Ruley, Genes Dev. 7: 535-545, 1993), also protected E1A-expressing cells from death following irradiation.

Similarly, $p53^{+/+}$ cells co-expressing E1A and T24 H-ras were extremely sensitive to low concentrations of 5-fluorouracil, etoposide, and adriamycin, as shown in FIG. 2. Viability of the untransfected MEFs and $p53^{+/+}$ and $p53^{-/-}$ clones co-expressing E1A and T24 H-ras was estimated by FITC uptake and FACS analysis 24 hours after treatment with the indicated concentrations of 5-fluorouracil (panel A), etoposide (panel B), and adriamycin (panel C). Each point represents the average and standard deviation obtained from at least 3 independent clones. All values were normalized to the relative viability of the corresponding untreated controls from the same experiment (generally greater than 90%). Closed circles represent untransfected $p53^{+/+}$ MEFs; open circles untransfected $p53^{-/-}$ MEFs; closed squares, $p53^{+/+}$ cells co-expressing E1A and T24 H-ras; open squares, $p53^{-/-}$ cells co-expressing E1A and T24 H-ras.

Cell death required p53, since $p53^{-/-}$ cells co-expressing E1A and T24 H-ras were largely resistant to these treatments. The differences in concentrations required for half-maximal killing of $p53^{+/+}$ and $p53^{-/-}$ cells

co-expressing E1A and T24 H-ras were greater than 20-fold in this assay. No decrease in viability was observed in the untransfected MEFs of either p53 genotype following exposure to 5-fluorouracil and etoposide, even at doses as high as 100 μM 5-fluorouracil. However, the viability of $p53^{-/-}$ cells co-expressing E1A and T24 H-ras began to decline at higher concentrations of these two drugs, and the viability of all cells declined with increasing concentrations of adriamycin. Thus, at sufficiently high concentrations, these agents can cause cell death and have chemotherapeutic value in a p53-independent manner.

During apoptosis, loss of membrane integrity is typically preceeded by chromatin condensation and internucleosomal cleavage of genomic DNA (Wyllie, Nature 284: 555-556, 1980). As visualized by staining with 2,4-diamidino-2-phenylindole (DAPI), significant numbers of $p53^{+/+}$ cells co-expressing E1A and T24 H-ras contained condensed chromatin and fragmented nuclei within 8 hours of treatment with the various anticancer agents. In contrast, $p53^{-/-}$ populations rarely contained cells with altered chromatin structure. Low molecular weight DNA from cells exposed to ionizing radiation or chemotherapy treatment was analyzed.

Low molecular weight DNA was isolated from $2 \times 10^6$ cells 24 hours after exposure to ionizing radiation or treatment with chemotherapeutic agents. Cells were irradiated with 5 Gy (+) or incubated in media containing 1 μM 5-fluorouracil (5-FU), 0.2 μM etoposide (ETOP), or 0.1 μg/ml adriamycin (ADR). DNA was resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

After irradiation, $p53^{+/+}$ cells co-expressing E1A and T24 H-ras contained large amounts of low molecular weight DNA, which produced a characteristic "ladder" on agarose gels. The degraded DNA was present in oligomers that were multiples of approximately 180-200 base pairs, suggesting

internucleosomal cleavage. Cells lacking p53 or expressing E1B did not contain degraded DNA after exposure to ionizing radiation. Treatment with low doses of 5-fluorouracil, etoposide, and adriamycin also induced DNA fragmentation in $p53^{+/+}$ cells co-expressing E1A and T24 H-ras, but not in p53-deficient cells. These data suggest that cell death in E1A-expressing cells resulted from common cellular response (apoptosis) to the various agents rather than from the direct genotoxicity of the treatments themselves.

Example 9 - p53 is Not Required For Cell Death Following Treatment With Sodium Azide

Since cells expressing E1A undergo p53-dependent apoptosis following serum depletion, irradiation, and treatment with various chemotherapeutic compounds, it was determined whether co-expression of p53 with E1A made cells sensitive to any toxic treatment. Untransfected MEFs and various clones co-expressing E1A and T24 H-ras were treated with sodium azide, an electron transport poison. The viability of the cells is shown in FIG. 3. Cell lines co-expressing E1A and T24 H-ras (squares) and the untransfected MEFs (circles) were incubated in various concentrations of sodium azide. $p53^{+/+}$ represent closed symbols, $p53^{-/-}$, open symbols. Cell viability was estimated by uptake of FITC and FACS analysis 12 hours after initiating treatment. Each data point represents the average of 2 independent clones.

Sodium azide-treated cells displayed a similar dose-dependent decrease in viability whether or not they expressed endogenous p53, although untransfected fibroblasts were less sensitive than cells co-expressing E1A and T24 H-ras. These results show that sodium azide-induced death did not require p53 function. Thus, not all cytotoxic agents require p53 function to be cytotoxic.

Example 10 - E1A Expression Allows Cells To Bypass p53-dependent Growth Arrest Following Irradiation

The effects of E1A and p53 expression on cell cycle progression following exposure to radiation and chemotherapeutic agents was determined. Cell cycle progression was assessed in cells exposed to ionizing radiation or 5-fluorouracil by 5-bromo-2'-deoxyuridine (BrdU) incorporation and measurement of cellular DNA content. $p53^{+/+}$ and $p53^{-/-}$ MEFs and E1A-expressing derivatives were incubated with BrdU for 4 hours beginning 14 hours after treatment. This represents a period when growth of irradiated fibroblasts is maximally inhibited and when $p53^{+/+}$ cells expressing E1A have initiated apoptosis. The relative amounts of cells in each phase of the cell cycle were estimated from the overall DNA content and the percentage of cells synthesizing DNA during the 4 hour BrdU pulse, as summarized in Table 3.

TABLE 3

CELL CYCLE PROGRESSION FOLLOWING EXPOSURE TO IONIZING RADIATION

| Treatment | Cells | p53 Genotype | % OF TOTAL G0/G1 | S | G2/M | %BrdU |
|---|---|---|---|---|---|---|
| none | MEF | +/+ | 61+/-3 | 17+/-4 | 22+/-5 | 28+/-4 |
| | E1A + ras | +/+ | 34+/-3 | 47+/-3 | 19+/-1 | 74+/-1 |
| | MEF | -/- | 40+/-5 | 21+/-9 | 39+/-4 | 46+/-13 |
| | E1A | -/- | 27+/-1 | 52+/-2 | 21+/-2 | 79+/-1 |
| | E1A + ras | -/- | 23+/-2 | 62+/-2 | 15+/-0 | 89+/-2 |
| 5Gy | MEF | +/+ | 70+/-1 | 4+/-2 | 27+/-1 | 6+/-2 |
| | E1A + ras | +/+ | 22+/-2 | 16+/-3 | 62+/-4 | 45+/-3 |
| | MEF | -/- | 35+/-3 | 18+/-5 | 47+/-2 | 43+/-13 |
| | E1A | -/- | 15+/-2 | 22+/-1 | 63+/-2 | 70+/-5 |
| | E1A + ras | -/- | 19+/-6 | 33+/-6 | 48+/-10 | 57+/-4 |

Untransfected MEFs and various clones were treated with 5 Gy ionizing radiation and incubated with BrdU. Cell proliferation was assessed by DNA content (propidium iodide staining) and incorporation of BrdU (using a FITC-anti-BrdU

antibody) by multiparameter flow cytometry analysis. The percentage of cells in each phase of the cell cycle was estimated by computer analysis of the propidium iodide staining data. The number of cells synthesizing DNA during the 4 hour pulse was estimated from the amount of BrdU incorporation. The data represents the average and standard deviation from 3 independent experiments.

A 5-fold decrease in cells incorporating BrdU was observed in $p53^{+/+}$ MEF cultures treated with ionizing radiation, and cells arrested predominantly in $G_0/G_1$. Irradiated $p53^{-/-}$ MEFs continued to synthesize DNA and accumulated in $G_2/M$, consistent with the involvement of p53 in radiation-induced $G_1$ (but not $G_2$) arrest. Treatment of wild type MEFs with 5-fluorouracil also caused an approximately 4-fold reduction in BrdU incorporation, with cells arresting predominantly in $G_1$, while $p53^{-/-}$ MEFs treated with 5-fluorouracil continued to incorporate BrdU and accumulated in $G_2/M$.

Cells expressing E1A continued to proliferate into S phase following treatment with ionizing radiation or 5-fluorouracil whether or not they expressed endogenous p53. E1A prevented $p53^{+/+}$ cells arresting in $G_1/G_0$ following irradiation. However, neither E1A nor the combination of E1A and T24 H-ras prevented irradiated cells from accumulating in $G_2/M$.

Example 11 - Identifying Agents That Are Cytotoxic Using Cell Lines Expressing E1A and Ras

The assays described in this example utilize cells derived from "knock-out" mice, as obtained in Example 1, following gene transfer to determine effective killing by putative anticancer agents. They compare oncogenically transformed cell lines derived from wild type mouse embryo fibroblasts (MEFs), or MEFs deficient for a specific gene, for example the tumor suppressor p53 gene. Thus, differences in cellular response to the agent are attributable to the

disrupted gene's activity. Cytotoxicity of the agent between the oncogenically transformed cell lines and the parental MEFs (analogous to "normal," i.e., wild type cells) are compared. These assays identify agents that require p53, act independently of p53, or bypass p53 function.

The cytotoxicity of various putative anticancer agents is tested in cells transformed by E1A and T24 H-ras derived from either $p53^{+/+}$ or $p53^{-/-}$ MEFs. To determine whether an agent acts through p53, dose-response curves are performed on cells and cell viability is determined. Untransfected (early passage) MEFs expressing or lacking p53 are used as controls. Any one or combination of four assays is used as described below: a multiwell plate viability assay, a colony regression assay, a plating assay, and a progression or regression assay for tumors in animals.

A. Multiwell Plate Viability Assay

This assay is the easiest of the four assays and preferably is used for large screens. The assay uses 24-well plates and allows testing of one drug at five different concentrations, as shown in FIG. 4. Cells obtained from Examples 1 and 2 are plated into each well at a density of $2-4 \times 10^3$ cells/well and allowed to adhere. The next day, cells are treated with various concentrations of a drug, and analyzed for viability after approximately 24 hours. Dose response curves are generated plotting cell viability vs. drug concentration. Cell viability is determined by standard methods using a colorimetric assay, or vital dye exclusion or chromium release. (Clarke et al., Nature 362: 849-852, 1993; Vanhaesebroeck et al., Oncogene 8: 1075-1081, 1993; Duerkson-Hughes et al., J. Immunol. 143: 4193-4200, 1993). Values are normalized to the viability of untreated cells. The drug 5-fluorouracil (which kills by a p53-dependent mode) is included as a control.

Hypothetical results are shown in FIG. 5. The closed circles represent untransfected $p53^{+/+}$ MEFs; open circles, untransfected $p53^{-/-}$ MEFs; closed squares, $p53^{+/+}$ cells

co-expressing E1A and T24 H-ras; and open squares, $p53^{-/-}$ cells co-expressing E1A and T24 H-ras. The panels of the figure represent different patterns for each of the tested cell types as compared to each other. Depending upon the pattern obtained for any given putative drug that is tested, an identification is made as to whether the tested agent is an anticancer agent, whether it is a p53-dependent or p53-independent anticancer agent, and whether it also kills normal cells.

In panel A, a p53-dependent mode of killing, with normal cells being resistant to the treatment is shown. In panel B, a p53-independent mode of killing, with normal cells being resistant to the treatment is shown. In panel C, a p53-independent mode of killing, with normal cells being susceptible to killing by the treatment is shown.

B. Colony Regression Assay

Unexpanded colonies transformed by E1A and ras are analyzed. Advantages of this assay are: (i) the ability to observe the effects of putative anticancer agents prior to significant expansion in cell culture, thereby minimizing cell culture selection for resistant phenotypes, (ii) the ability to monitor large numbers of independent clones to assess the effects of clonal variation.

2-3 weeks after transfection of cells with plasmids expressing E1A and ras oncogenes, $p53^{+/+}$ and $p53^{-/-}$ colonies are marked and transferred to growth medium (without Hygromycin B) supplemented with various concentrations of the anticancer agent to be tested. The expansion or regression of colonies is monitored over a few day period. Specific regression of $p53^{+/+}$ colonies indicates a p53-dependent mode of death.

C. Plating Assay

This assay measures the long term growth potential of cells treated with a putative anticancer agent. Cells not immediately killed by a drug may lose reproductive potential and therefore be unable to proliferate at clonal densities.

Drugs that inhibit the reproductive potential of these cells reduce their ability to form colonies when plated at clonal density.

$p53^{+/+}$ and $p53^{-/-}$ cells transformed by E1A and T24 H-ras are plated at clonal density, approximately $10^3$ cells/100 mm plate, in medium containing various concentrations of the agent to be tested. Medium is changed every 3-4 days until the colonies are large enough to be observed without magnification. Plates are fixed in formaldehyde and stained with crystal violet to assist in counting colonies. Values are normalized to the plating efficiency of untreated cells from the same cell line (# of colonies in drug/# colonies untreated). Drugs with known modes of action are used as controls.

Hypothetical results are shown in FIG. 6. Depending upon the pattern of results obtained for any given putative drug that is tested, a determination of its anticancer properties are made. The closed squares represent $p53^{+/+}$ cells co-expressing E1A and T24 H-ras; open squares, $p53^{-/-}$ cells co-expressing E1A and T24 H-ras. In panel A, a p53-dependent mode of killing (or inhibition of reproductive potential) is shown, and in panel B, a p53-independent mode is shown.

D. Progression or Regression Assay of Tumors in Animals

Cells transformed by E1A and T24 H-ras are highly tumorigenic. Therefore, these cells can be used to generate tumors in animals for tests of drug effectiveness in vivo as well as initial estimates of drug toxicity in the animal. Since the MEFs used to generate the cell lines differ only in the status of the disrupted apoptotic gene, differences in tumor response to therapy are attributable to the disrupted gene.

For p53, cellular sensitivity to p53-dependent apoptosis in cells expressing E1A and ras is not selected against during tumor development in nude mice, suggesting that tumors can be generated that are either sensitive or resistant to

certain anticancer agents. Tumor progression/regression can be measured using a variety of methods known to those skilled in the art. (Trail et al., Science 261: 212-215, 1993). One example is described below.

Cells transformed by E1A and T24 H-ras derived from either $p53^{+/+}$ or $p53^{-/-}$ MEFs are infected subcutaneously into nude mice ($2 \times 10^6$ cells/injection). (Swiss nu/nu obtained from Taconic, Germantown, New York). Tumors begin to appear within 1-3 weeks. At defined points thereafter, animals are treated (or left untreated as a control) with a putative anticancer agent. The method of treatment depends upon the agent being tested, and includes any appropriate mode, e.g., orally, intravenously, subcutaneously, intraperitoneally, topically, combined with a liposome, in a time release formulation, transgenically, or by irradiation. Tumors are monitored for growth, regression, or reoccurrence at various times thereafter, using standard methods. (Trail et al., Science 261: 212-215, 1993).

E. Interpretation of Results of Assays

i. Identifying Drugs that Require p53 Function for Cytotoxicity

A drug that acts via a p53-dependent mechanism kills $p53^{+/+}$ cells expressing E1A and ras more readily than the equivalent $p53^{-/-}$ cells. For example, the concentration required to kill 50% of cells co-expressing E1A and ras (see FIG. 5, panel A), or reduce plating efficiency by 50% (see FIG. 6, panel A) is considerably lower for $p53^{+/+}$ cells than for $p53^{-/-}$ cells. Moreover, this drug has minimal toxicity with respect to untransfected fibroblasts. Similarly, a p53-dependent drug induces regression of $p53^{+/+}$ tumors but has a minimal effect on $p53^{-/-}$ tumors. Any toxic side effects in the mouse at concentrations that generate tumor regression are determined in the assay for progression or regression of tumors in animals.

ii. Identifying Drugs that Do Not Require p53 Function for Cytoxicity

Not all forms of apoptosis require p53 function, (Clark et al., Nature 362: 849-852, 1993), suggesting that potentially useful drugs may activate apoptosis independently of p53 or "downstream" of p53 in the p53-dependent apoptotic pathway. Such a drug kills $p53^{+/+}$ and $p53^{-/-}$ cells expressing E1A and ras equally (see FIG. 5, panels B and C; FIG. 6, panel B). Moreover, this drug should have minimal toxicity with respect to untransfected fibroblasts (multiwell plate viability assay). Similarly, a p53-independent drug induces regression of both $p53^{+/+}$ and $p53^{-/-}$ tumors, with minimal toxicity at the active concentration (progression or regression assay of tumors in animals).

iii. Identifying Molecules that Bypass, Restore or Replace p53 Function

Anticancer agents that act by restoring, bypassing or replacing p53 activity can be identified by the methods of the invention. Hypothetical results using the plating assay are shown in FIG. 7. Depending upon the pattern of results obtained for any given putative drug that is tested, a determination of its anticancer properties is made. The closed squares represent $p53^{+/+}$ cells co-expressing E1A and T24 H-ras; open squares, $p53^{-/-}$ cells co-expressing E1A and T24 H-ras. Solid lines represent a p53-dependent drug (e.g. 5-fluorouracil) alone; dashed lines, a p53-dependent drug plus the drug that bypasses or restores p53 function. When the p53-independent drug is used in combination with a p53-dependent drug (e.g., 5-fluorouracil), the concentration of the 5-fluorouracil required to kill 50% of the p53-deficient cells expressing E1A and ras is reduced.

Such p53 drugs may also be tested using the progression or regression assay of tumors in animals to determine their usefulness in vivo.

Example 12 - Screening Tumor Biopsies for p53 Mutations

Tumor biopsies are screened for the presence or absence of functioning genes or molecules involved in apoptosis in order to establish more effective treatment regimens. For example, tumors are screened for a p53 mutation. For tumors containing p53 mutations, p53-dependent drugs would be less desirable or would have to be used at higher concentrations than drugs that act independently of p53 or trigger redundant pathways.

Both point mutations and gene deletions can contribute to loss of p53 function in vivo. The methodology for testing for the presence of a p53 mutation is known to those skilled in the art. Suitable methods include: (i) immunohistochemical analysis of tumors using mutant-specific antibodies (Davidoff et al., Surgery 110: 259-264, 1991; Thor et al., J. Nat'l. Cancer Inst. 84: 845-855, 1992; (ii) single strand conformational polymorphism analysis (SSCP) for detecting point mutations and loss of heterozygosity (D'Amico et al., Oncogene 7: 339-346, 1992); (iii) nondenaturing gradient gel electrophoresis (Thorlacius et al., Cancer Res. 53: 1637-1641, 1993); (iv) direct sequencing (Chiba et al., Oncogene 5: 1603-1610, 1990; Thorlacius et al., Cancer Res. 53: 1637-1641, 1993; D'Amico et al., Oncogene 7: 339-346, 1992); and (v) RNase protection assay (Chiba et al., Oncogene 5: 1603-1610, 1990).

Example 13 - Introduction of Wild Type p53 DNA Into p53-Deficient Cells

Restoration of the p53-dependent response to anticancer agents in p53 deficient cells is accomplished by introducing wild type p53 DNA into these cells. Introduction of wild type p53 DNA into p53-deficient cells is accomplished by retrovirus-mediated gene transfer (Yeargin et al., Leukemia 6: 855-915, 1992; Yeargin et al., J. Clin. Invest. 91: 211-217, 1993). The virus co-expresses a selectable marker so as to eliminate cells not acquiring exogenous DNA. The

cells are subjected to multiple rounds of infection at high multiplicity of infection (i.e., # of infectious units/cell), in order to transfect as many cells as possible. Incubation of cultured cells in the presence of polybrene during infection increases the infection frequency.

Alternatively, introduction of wild type p53 DNA into the p53-deficient cells is accomplished by gene transfer of p53-expressing plasmids. (Shaw et al., Proc. Nat'l. Acad. Sci. U.S.A. 89: 4495-4499, 1992; Baker et al., Science 249: 912-915, 1990).

The transfected cells are subjected to treatment with p53-dependent anticancer agents, or a combination of p53-dependent and p53-independent anticancer agents. Since the transfected cells contain a wild type p53 gene, a p53-dependent response to the anticancer agents is effected, e.g., a retardation of unwanted cell growth and/or reproduction, destruction of cells, or a loss of viability or unwanted characteristics.

Example 14 - Introduction of Wild Type p53 DNA into Mice with Tumors Lacking p53

Mice lacking tumor suppressor gene p53 have been generated (Donehower et al., Nature 356: 215-220, 1992; Clarke et al., Nature 362: 849-852, 1993), and are available as described in Example 1. Mice homozygous for p53-deficiency develop spontaneous tumors primarily of thymic origin, i.e., T cell lymphoma (Donehower et al., Nature 356: 215-220, 1992). Furthermore, mice heterozygous for p53-deficiency develop a wide range of tumors (at later times than the homozygous mice), which are similar to humans with Li-Fraumeni syndrome (Malkin et al., N. Engl. J. Med. 326: 1309-1315, 1992), and therefore provide a good model for human cancer. In tumors that develop from heterozygous mice, the wild type p53 gene is frequently lost or mutated.

These mice, or other "knock-out" mice with mutations in an apoptotic pathway, provide a useful system for determining

the effectiveness of gene therapy in treating spontaneous p53-deficient tumors by the introduction of a wild type p53 gene.

The wild type p53 gene is introduced into spontaneous p53-deficient tumors so as to enhance the effectiveness of anticancer agents in such tumors. The mouse carrying the tumor is $p53^{-/-}$ or $p53^{+/-}$. This introduction is accomplished using a p53-expressing retrovirus injected into the mouse, preferably into a vessel of the mouse that supplies the tumor. The effectiveness of p53-dependent anticancer agents in causing regression or cures of tumors in mice given a p53 gene is tested by comparisons to untreated mice, and to control mice which are given a retrovirus not expressing p53.

Example 15 - Testing p53-Independent Anticancer Agents in Mice with Tumors Lacking p53

The mice described in Example 1 provide a useful system to assess the utility of the p53-independent drugs identified in Example 11, in treating spontaneous p53-deficient tumors in a whole organism. Since $p53^{+/-}$ animals develop tumors of varying tissue origin, these animals provide a good in vivo system for evaluating the utility of the drugs of this invention that act by a p53-independent mechanism.

At defined points, such mice with a spontaneous tumor are treated (or left untreated as a control) with a p53-independent agent identified as described in Example 11. The method of treatment depends upon the particular agent being tested, and includes any appropriate mode, e.g., orally, intravenously, subcutaneously, intraperitoneally, topically, combined with a liposome, in a time release formulation, transgenically, or by irradiation. Tumors are monitored for growth, regression, or reoccurrence at various times thereafter, using standard methods. (Trail et al., Science 261: 212-215, 1993).

Example 16 - In Vivo Tumor Response to Anti-Cancer Therapies Depends Upon the Presence of p53

This example illustrates that the presence of p53 has a dramatic effect on tumor response to γ-irradiation and chemotherapy in vivo.

A transplantable fibrosarcoma model was developed to investigate the effects of p53 on tumor growth and sensitivity to drug and radiation therapy in vivo. Embryonic fibroblasts derived from wild type mice and mice which lack functional p53 genes were oncogenically transformed by the combination of adenovirus early region 1A (E1A) and an activated ras oncogene (T24-H ras). These cells can form tumors following subcutaneous injection into nude mice regardless of their p53 status, but cells which express p53 remain sensitive to apoptosis in vitro. Lowe et al., Proc. Natl. Acad. Sci. U.S.A. 91:2026 (1994); Lowe et al., Cell 74: 957-967 (1993). This oncogene combination provides a well-characterized system of multi-step carcinogenesis (Ruley, Nature 304: 602 (1983)), analogous to many naturally occurring tumors. Since these cells are highly oncogenic, tumor growth can occur without strong selection for additional mutations. It was therefore possible to compare tumors that differed primarily in their p53 status.

Oncogenically transformed cells expressing p53 formed fewer tumors, and with a longer latency, than p53-deficient cells. These results were in agreement with earlier studies. Lowe et al., Proc. Natl. Acad. Sci. U.S.A. 91:2026 (1994). Cells lacking p53 gave rise to tumors at all injected sites compared to 82 $\pm$ 24% ($p<0.11$) of sites injected with p53-expressing cells, with an average latency of 8 $\pm$ 4 and 18 $\pm$ 7 days, respectively ($p<0.03$). However, after reaching a palpable size, tumors derived from both wild type and p53-deficient cells expanded at similar rates (Fig. 8), until the tumors became sufficiently large (>1 $cm^3$) that the animals were sacrificed.

Table 4 and Fig. 8 show tumor response to radiation and chemotherapy in the animals injected with p53-expressing (+/+) and p53-deficient (-/-) cells. Athymic nude mice were injected with embryonic fibroblasts transformed by E1A and an activated ras oncogene (T24 H-ras) (Lowe et al., Proc. Nat'l Acad Sci. USA 91: 2026 (1994), and tumor volumes were monitored as follows. Minimally passaged $p53^{+/+}$ and $p53^{-/-}$ mouse embryonic fibroblasts transformed by E1A and T24 H-ras were detached from tissue culture plates, washed, and resuspended in phosphate buffered saline (PBS). 2 x $10^6$ cells were injected into both the left and right flank of athymic nude mice (age 4-8 weeks). Upon reaching a palpable size, tumor volumes were estimated from caliper measurements of tumor length (L) and width (l) according to the formula: $((L \times l^2)/2)$. In general, tumors were allowed to expand to 0.15-0.5 $cm^3$ prior to treatment. Animals receiving lower doses of radiation ($\leq$7 Gray (Gy)) were given a single fraction to the whole body in a Gammacell 40 irradiator containing a $^{137}Cs$ source (approximately 0.8 Gy/min.) For higher doses, a lead shield was used to reduce radiation toxicity to the bone marrow and thoracic cavity. Adriamycin was injected intraperitoneally at a dose of 10 mg/kg in PBS.

As shown in Fig. 8, at the indicated times (arrows), animals harboring tumors derived from p53-expressing (panels A,C,E) and p53-deficient (B,D,F) cells were irradiated with either 7 Gy (A,B) or 12 Gy (C,D), or treated with adriamycin (E,F). Fig. 8 shows representative examples of tumors derived from p53-expressing clones C1 (open circles), C8 (closed circles in A and E), and C6 (closed circles in C), and p53-deficient clones A4 (open triangles), A9 (closed triangles in B and D), and E6 (closed triangles in F).

These results indicate that p53 had a dramatic effect on tumor response to both γ-irradiation and adriamycin treatment. As illustrated in FIG. 8, most tumors derived from p53-expressing cells responded well to 7 Gy, typically

TABLE 4

SUMMARY OF TUMOR RESPONSE TO RADIATION AND CHEMOTHERAPY

| Clone | p53 | Treat-ment | n | Tumor Response Min.Vol. | d | Vol.(d7) |
|---|---|---|---|---|---|---|
| C1 | (+/+) | 7Gy | 2 | 53 | 6 | 53 |
| | | 9Gy | 2 | 40 | 7 | 40 |
| | | 12Gy | 4 | 11±10 | 7 | 10 |
| | | Adr | 2 | 42 | 3 | NA |
| C6 | (+/+) | 7Gy | 2 | 50 | 3 | 89 |
| | | 9Gy | 3 | 42±27 | 4 | 67 |
| | | 12Gy | 2 | 20 | 4 | 37 |
| | | Adr | 2 | 13 | 6 | NA |
| C8 | (+/+) | 2Gy | 3 | 29±12 | 8 | 64 |
| | | 5Gy | 3 | 8±13 | 5 | 12 |
| | | 7Gy | 3 | 16±9 | 7 | 17 |
| | | Adr | 6 | 19±13 | 6 | 15 |
| C10 | (+/+) | 5Gy | 2 | 96 | 1 | 133 |
| | | 7Gy | 2 | 82 | 5 | 117 |
| | | 12Gy | 2 | 89 | 3 | 95 |
| | | Adr | 1 | 100 | 0 | 186 |
| A4 | (-/-) | 7Gy | 2 | 100 | 0 | 230 |
| | | 12Gy | 2 | 100 | 0 | 305 |
| | | Adr | 4 | 100±0 | 0 | 289 |
| A8 | (-/-) | 7Gy | 4 | 91±13 | 0 | 177 |
| | | 12Gy | 2 | 100 | 0 | 125 |
| A9 | (-/-) | 7Gy | 2 | 95 | 1 | 223 |
| | | 12Gy | 4 | 100±0 | 0 | 171 |
| E6 | (-/-) | 7Gy | 2 | 100 | 0 | 177 |
| | | Adr | 4 | 100±0 | 0 | 232 |

NA, not applicable

regressing to less than 50% of their pretreatment volume within 5 days. Tumors derived from p53-deficient cells generally continued to grow, even following doses as high as 12 Gy. Similar results were obtained when animals harboring tumors were treated with adriamycin. Therefore, the presence of wild type p53 was required for efficient tumor regression following radiation or chemotherapy.

This data is summarized in Table 4. Animals harboring tumors derived from p53-expressing (+/+) or p53-deficient (-/-) cells were treated on day 0 with the indicated dose of ionizing radiation (in Gy) or adriamycin (Adr), as described above. The maximum response is represented by the smallest tumor volume (minimum volume) occurring after treatment (as a percentage of the tumor volume on day 0) and the day (d) at which the minimum volume was achieved. The tumor volume at day 7 (d7) post-treatment is listed as a percentage of the volume on day 0. Values represent the average of two or more experiments. Standard deviations are shown where applicable. "n" is the number of tumors analyzed for a given clone and treatment.

Some of the tumors derived from p53-expressing cells disappeared completely, although all but one eventually regrew (not shown). The magnitude of the response varied depending on the clone injected. For example, tumors derived from one p53-expressing clone (C8) responded well to 2 Gy while another clone (C10) responded poorly to doses as high as 12 Gy.

In contrast, tumors derived from p53-deficient cells displayed little, if any, response to 7 Gy of ionizing radiation. Although some tumors remained stationary for several days, 9 of 10 tumors were significantly larger 1 week following treatment (see Fig. 8 for representative curves and Table 4 for a summary of the averaged data). In several instances, radiation treatment had no effect on tumor growth (Fig. 8, panel B). While tumors derived from p53-expressing

cells were even more sensitive to higher doses of radiation, p53-deficient tumors were refractory to levels as high as 12 Gy (Fig. 8, panels C and D, and Table 4).

p53 also influenced the effectiveness of chemotherapy. Specifically, adriamycin treatment induced a rapid regression of tumors derived from wild type cells but did not significantly affect the growth of p53-deficient tumors (Fig. 8, panels E and F). The amount of adriamycin used in these experiments approached the maximum tolerated dose for a single injection (Scheulen et al., Strahlentherapie Onkologie 165:529 (1989)). Thus, while higher doses of adriamycin are capable of killing p53-deficient cells in vitro (Lowe et al., Cell 74: 957 (1993), the concentrations required for this effect are toxic to the animal. The one tumor derived from p53-expressing cells that failed to respond to adriamycin was derived from the same clone that was relatively radioresistant (C10, Table 4).

Example 17 - In Vivo Regression of Tumors Derived From p53-Expressing Cells Subjected to Anti-Cancer Therapies Is Due to Apoptosis

This example illustrates that the regression of tumors derived from p53-expressing cells subjected to γ-irradiation or chemotherapy is due to apoptosis, and that p53-deficient tumors which do not regress are less susceptible to apoptosis.

In general, cells that undergo apoptosis are interspersed throughout tumors and have distinct morphological features, including cell shrinkage, chromatin condensation, and loss of extracellular contacts. Kerr et al., Br. J. Cancer 26: 239 (1972). Apoptotic cells often activate an endonuclease that breaks genomic DNA (Wyllie, Nature 284: 555 (1980)), generating products which are readily detected in situ using the terminal deoxytransferase-mediated dUTP-biotin nick end labeling (TUNEL) method. Gavrieli et al., J. Cell Biol. 119: 493 (1992).

Untreated tumors derived from wild type and p53-deficient fibroblasts described in Example 16, were shown to display a typical fibrosarcoma histology. Both tumor types contained many mitotic figures, indicating a high percentage of proliferating cells. Both also contained some necrotic zones. However, untreated tumors derived from p53-expressing cells displayed more apoptosis compared to p53-deficient tumors, as assessed by both histology and the TUNEL assay.

Cell death in tumors receiving radiation or adriamycin treatment as described in Example 16, had morphological and physiological features of apoptosis. Histological analysis was done on untreated tumors or tumors recovered 40-48 hours following the anti-cancer treatment. Hematoxylin/eosin-staining of tumor sections was done from (A) an untreated tumor derived from a p53-expressing clone (1000X); (B) an untreated p53-deficient tumor (1000x); (C) and (E) tumors derived from p53-expressing cells following adriamycin treatment (1000X and 200X, respectively); (D) and (F) p53-deficient tumors following adriamycin treatment (1000X and 200X, respectively). Treatment with ionizing radiation produced similar results. Sections from p53-expressing (A, C) and p53-deficient (B, C) tumors, either untreated (A, B) or 40-48 hours following treatment with 7 Gy ionizing radiation (C, D), were subjected to TUNEL staining (Gavrieli et al., J. Cell Biol. 119:493 (1992)). TUNEL-positive cells were visualized using a horseradish peroxidase-based detection method that stained cells dark brown. Tissue sections were counterstained with methyl green and photographed at 200X.

Tumors derived from p53-expressing cells contained many pyknotic cells interspersed throughout the tumor within 2 days after treatment. Many of the pyknotic cells contained fragmented nuclei and stained with TUNEL, and the remaining normal cells were often surrounded by large regions of extracellular space. By contrast, p53-deficient tumors

treated with either γ-irradiation or adriamycin contained few regions of pyknosis and cell loss. Nevertheless, there was a small but reproducible increase in the number of TUNEL-positive cells interspersed throughout the tumor. These data indicate that the regression of tumors derived from p53-expressing cells was due to apoptosis, and that p53-deficient tumors, which did not regress were less susceptible to apoptosis.

Example 18 - Tumors That Become Resistant to Anti-Cancer Therapies In Vivo Can Result From a p53 Mutation

This example illustrates that initially responsive tumors derived from p53-expressing clones display a reduced response to subsequent treatments and several become completely resistant.

Moreover, tumors derived from one p53-expressing clone (C10) responded poorly to the initial treatments with radiation and chemotherapy (Fig. 9 and Tables 4 and 5), even though the injected cells readily underwent apoptosis in vitro (not shown).

Since an intact p53-dependent apoptotic program is essential for significant tumor regression, it was tested whether acquired resistance involved loss of p53 function by mutation. Regions of tumor cDNAs corresponding to exons 5-8 of the p53 gene were amplified by polymerase chain reaction (PCR) and sequenced. This region contains greater than 90% of the mutations responsible for p53 inactivation in human tumors. Hollstein et al., Science 253:49 (1991). Amplification and sequences were performed as follows. Tumor cells were dispersed with trypsin and cultured in medium containing 50 μg/ml hygromycin B to select for tumor-derived cells (which contain hygromycin phosphotransferase, Lowe et al., Proc. Natl. Acad. Sci. U.S.A. 91:2026 (1994)). RNA was obtained from the tumor cells maintained for less than about one week in culture. For tumor 915L, RNA was obtained directly from frozen tumor

TABLE 5

p53 MUTATIONS IN RECURRENT AND RESISTANT TUMORS

| Clone | Tumor Response: Tumor | Tumor Response: Treatment (min.vol.) | Mutation: Nucleotide | Mutation: Murine | Mutation: Human | Mutation: Amino Acid |
|---|---|---|---|---|---|---|
| C1 | 970L | 9(32);9(74) | ND | NA | NA | NA |
| | 970R | 9(48);9(100) | ND | NA | NA | NA |
| C6 | 975L | 9(35) | TTC>TGC | 131 | 134 | phe>cys |
| | 977L | 12(21) | TTC>TGC | 131 | 134 | phe>cys |
| | 977R | 12(19) | TTC>TGC | 131 | 134 | phe>cys |
| | | | TGC>GGC | 239 | 242 | cys>gly |
| C8 | 396L | 7(11) | ND | NA | NA | NA |
| | 380L | 5(0);5(41) | ND | NA | NA | NA |
| | 912R | 2(33);2(45); 2(74);2(100) | ND | NA | NA | NA |
| | 929L | A(6);2(39); 2(42);2(74); 2(100) | ND | NA | NA | NA |
| C10 | 934 | none | CAC>CGC** | 211 | 214 | his>arg |
| | 933R | 2Gyx7*(78) | CAC>CGC** | 211 | 214 | his>arg |
| | 915L | 7(63)7(100) | CAC>CGC** | 211 | 214 | his>arg |

ND, not detected
NA, not applicable
*treatment consisted of 7 daily doses of 2Gy
**no wild type sequence detected

tissue. cDNA was prepared by standard methods using a primer corresponding to sequences within either exon 9 or 11 of murine p53. Bienz et al., Embo J. 3:2179 (1984). Amplification of p53 sequences corresponding to exons 5 to 8 was accomplished by PCR with primers specific to sequences within exons 4 and 9. Sequencing was carried out using several primers specific to exons 5-8. Mutations were verified by sequencing the opposite strand or by repeating the sequencing. The codon 132 mutation in tumors 975L, 977L, and 977R created a BsoF1 site, allowing independent verification of the mutation by restriction digestion of PCR products.

The results are shown in Table 5. For each tumor, the treatment(s) are indicated as the dose of ionizing radiation (in Gy) or as adriamycin treatment (A). In parentheses is the minimum volume achieved following treatment, normalized to the tumor volume on the day of treatment. Also shown is the detected nucleotide change, the affected codon in murine p53 (Bienz et al., Embo J. 3:2179 (1984)), the corresponding human codon (Hollstein et al., Science 253:49 (1991)), and the predicted amino acid substitution.

As summarized in Table 5, p53 mutations were detected in approximately 50% of the resistant or relapsed tumors, at codons observed mutated in human cancer. Hollstein et al., Science 253:49 (1991). One untreated tumor and two nonresponsive tumors derived from clone C10 acquired a missense mutation (histidine to arginine) at codon 211. No wild type sequence was detected in these tumors, suggesting that they had lost the normal p53 allele. Although not detectable in the injected population, cells harboring this mutation presumably represented a small percentage of the injected cells and were selected during tumor growth. This probably accounts for the poor response of all tumors derived from clone C10.

In tumors arising from clone C6, p53 mutations were detected in recurrent tumors that were previously treated with high levels of ionizing radiation (Table 2). While no p53 mutations were detected in the injected cells (not shown), a phenylalanine to cystine substitution was observed at codon 131 in each relapse tumor analyzed, again suggesting that a small percentage of parental cells harbored this mutation. A second missense mutation (cystine to serine at codon 239) was observed in only one C6-derived tumor (#977R), implying it was acquired during tumor expansion.

Fig. 9 shows the acquired resistance of tumors derived from p53-expressing clones. Athymic nude mice were injected with transformed embryonic fibroblasts (time 0) and treated as described in Fig. 8. (A) Animals harboring tumors derived

from either clone C1 (open circles) or C8 (closed circles) were treated with multiple doses of ionizing radiation (2 Gy/dose and 9 Gy/dose, respectively) at the times indicated by the arrows. (B) Tumors arising from p53-expressing clone C10 were treated with either ionizing radiation (7 Gy, triangles; 12 Gy, closed circles) or adriamycin (10 mg/kg, open circles) as indicated (arrows).

In vivo response of tumor 970R is shown in Fig. 9, panel B (open circles); 977L in Fig. 8, panel C (open circles); 396L in Fig. 8, panel A (closed circles); 912R in Fig. 9, panel B (closed circles); and 915L in Fig. 9, panel A (triangles).

The data depicted in the above described table and figures suggest that ionizing radiation selectively eliminated tumor cells that expressed only wild type p53, leading to the enrichment of cells harboring p53 mutations. However, p53 mutations were not detected in several recurrent tumors, including some that demonstrated complete resistance to additional therapy (Table 5). It is possible that some mutations were not detected by the limited analysis performed, or involved other genes in the p53 pathway. Alternatively, p53-independent mechanisms may also give rise to radioresistant tumors. The mdm-2 oncogene, which can inactivate p53 and is amplified in many human sarcomas, was not overexpressed in any of the tumors tested.

Those skilled in the art will be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for evaluating the ability of a treatment to adversely affect the growth or viability of a cell, comprising:

providing a cell having a first condition which reduces the susceptibility of said cell to apoptosis;

administering said treatment to said cell; and

determining whether said treatment affects the value of a parameter related to the growth or viability of said cell.

2. The method of claim 1 wherein said cell is obtained from a cell culture.

3. The method of claim 1 wherein said cell is a mouse embryo fibroblast.

4. The method of claim 1 wherein said cell is part of an organism.

5. The method of claim 4 wherein said organism is an animal, said animal selected from the group consisting of an animal being homozygous for a mutation in the apoptotic pathway, an animal being heterozygous for a mutation in the apoptotic pathway, and an animal being wild type for the apoptotic pathway.

6. The method of claim 5 wherein said animal is a transgenic animal.

7. The method of claim 1 wherein said first condition includes a mutation in a gene that affects the apoptotic pathway.

8. The method of claim 7 wherein said mutation is homozygous.

9. The method of claim 7 wherein said mutation is heterozygous.

10. The method of claim 7 wherein said mutation is in a tumor suppressor gene.

11. The method of claim 10 wherein said tumor suppressor gene is p53.

12. The method of claim 7 wherein said mutation is in a regulator gene for the p53 tumor suppressor gene.

13. The method of claim 1 wherein said first condition includes a nonwild type level of expression of a gene which affects the apoptotic pathway.

14. The method of claim 1 wherein said first condition comprises the presence of an oncogene product which inhibits apoptosis.

15. The method of claim 14 wherein said oncogene is selected from the group consisting of bcl2, HPV-E6 and adenovirus E1B.

16. The method of claim 1 wherein said cell further comprises a second condition which in a wild type background enhances the susceptibility of said cell to apoptosis.

17. The method of claim 16 wherein said second condition comprises an expressed oncogene.

18. The method of claim 17 wherein said oncogene is the adenovirus E1A gene.

19. The method of claim 17 wherein said oncogene is the c-myc gene.

20. The method of claim 16 wherein said cell further comprises a third condition which allows establishment of a permanent cell line when said second condition is present and comprises an expressed oncogene.

21. The method of claim 20 wherein said third condition allows said cell to form a tumorigenic cell line.

22. The method of claim 20 wherein said third condition comprises an expressed oncogene.

23. The method of claim 22 wherein said oncogene is T24 H-ras.

24. The method of claim 1 wherein said first condition is a mutation in the tumor suppressor gene p53, said second condition is an expressed adenovirus E1A gene, and said third condition is an expressed T24 H-ras gene.

25. The method of claim 1 further comprising administering a second treatment to said cell which in the presence of tumor suppressor gene p53 activity would reduce the growth or viability of said cell.

26. The method of claim 25 wherein said second treatment is selected from the group consisting of a chemotherapeutic agent and radiation.

27. A method for identifying an agent useful for treating unwanted cell proliferation, comprising:

providing a cell comprising a first condition which reduces susceptibility of said cell to apoptosis and a second condition which in a wild type background enhances susceptibility of said cell to apoptosis;

administering said agent and determining if said agent affects the growth rate of said cell, a decrease in said growth rate being correlated with the ability of said agent to treat said unwanted cell proliferation.

28. The method of claim 27 further comprising said cell including a third condition which allows establishment of a permanent cell line when said second condition comprises an oncogene.

29. The method of claim 28 wherein said third condition allows said cell to form a tumorigenic cell line.

30. The method of claim 27 wherein said cell is a mouse embryo fibroblast.

31. The method of claim 27 wherein said first condition is homozygosity for a tumor suppressor gene p53 mutation, said second condition is the presence of the adenovirus E1A gene, and said third condition is the presence of the T24 H-ras gene.

32. A method for evaluating the ability of a treatment to affect the growth or viability of cells which are wild type for the apoptotic pathway, comprising:

providing a first cell which is wild type for the apoptotic pathway;

providing a second cell which has a mutation in the apoptotic pathway;

administering said treatment to said first cell and said second cell;

determining whether said treatment affects the value of a parameter related to the growth or viability of said first cell and said second cell; and

comparing the values of said growth or viability parameter between said first cell and said second cell such that if said treatment inhibits said parameter more extensively in said first cell than in said second cell, then the treatment is apoptotic pathway dependent and identifies the treatment as being effective for cells which are wild type for the apoptotic pathway.

33. The method of claim 32 wherein said mutation is in the tumor suppressor p53 gene.

34. The method of claim 32 wherein said mutation is homozygous.

35. A method of utilizing an anticancer drug to alleviate the symptoms of cancer in an organism, which anticancer drug has been originally selected as an anticancer agent by:

providing a cell having a first condition which reduces the susceptibility of said cell to apoptosis;

administering a first treatment to said cell; and

determining whether said first treatment affects the value of a parameter related to the growth or viability of said cell.

36. The method of claim 35 further comprising utilizing a second treatment selected from the group consisting of a chemotherapeutic agent and radiation to alleviate the symptoms of cancer in the organism.

37. A method of treating a cell which is tumorigenic, said cell being part of an organism, by administering tumor suppressor gene p53 DNA in an amount sufficient to give expression of a therapeutically effective amount of p53 protein.

38. The method of claim 37 wherein said cell is deficient in tumor suppressor gene p53 function.

39. The method of claim 37 further comprising the step of administering a treatment selected from the group consisting of a chemotherapeutic agent and radiation.

40. A method of treating a cell obtained from a tumorigenic cell culture, said cell being deficient for tumor suppressor gene p53 function, comprising:

administering tumor suppressor gene p53 DNA in an amount sufficient to give expression of a therapeutically effective amount of p53 protein; and

administering a therapeutically effective amount of a treatment selected from the group consisting of a chemotherapeutic agent and radiation.

41. A method of treating a cell which is tumorigenic, said cell being part of an organism, by administering a therapeutically effective amount of tumor suppressor gene p53 protein.

42. The method of claim 41 wherein said cell is deficient in tumor suppressor gene p53 function.

43. The method of claim 41 further comprising the step of administering a treatment selected from the group consisting of a chemotherapeutic agent and radiation.

44. A method of treating a cell obtained from a tumorigenic cell culture, said cell being deficient for tumor suppressor gene p53 function, comprising:

administering a therapeutically effective amount of tumor suppressor gene p53 protein; and

administering a therapeutically effective amount of a treatment selected from the group consisting of a chemotherapeutic agent and radiation.

45. A method of treating an organism with cancer, comprising:

testing said organism's cancer cells for the presence or absence of a tumor suppressor gene p53 mutation; and

if a p53 mutation is absent, then administering a therapeutically effective amount of a treatment selected from the group consisting of a p53-dependent treatment, and a combination of a p53-dependent and a p53-independent treatment; and

if a p53 mutation is present, then administering a therapeutically effective amount of a treatment selected from the group consisting of a p53-independent treatment, and a sufficiently high level of a normally p53-dependent treatment so as to overcome the p53-dependent property of said p53-dependent treatment.

46. The method of claim 45 wherein said screening is selected from the group consisting of immunohistochemical analysis of tumors using mutant-specific antibodies, single strand polymorphism analysis for detecting point mutations and loss of heterozygosity, nondenaturing gradient gel electrophoresis, direct sequencing, and RNase protection assays.

47. A mouse embryonic fibroblast cell, said cell being homozygous for a tumor suppressor gene p53 mutation and having an adenovirus E1A gene and a T24 H-ras gene.

48. A mouse embryonic fibroblast cell, said cell being heterozygous for a tumor suppressor gene p53 mutation and having an adenovirus E1A gene and a T24 H-ras gene.

49. A mouse embryonic fibroblast cell line comprising cells that are homozygous for a tumor suppressor gene p53 mutation and having an adenovirus E1A gene and a T24 H-ras gene.

50. A mouse embryonic fibroblast cell line comprising cells that are heterozygous for a tumor suppressor gene p53 mutation and having an adenovirus E1A gene and a T24 H-ras gene.

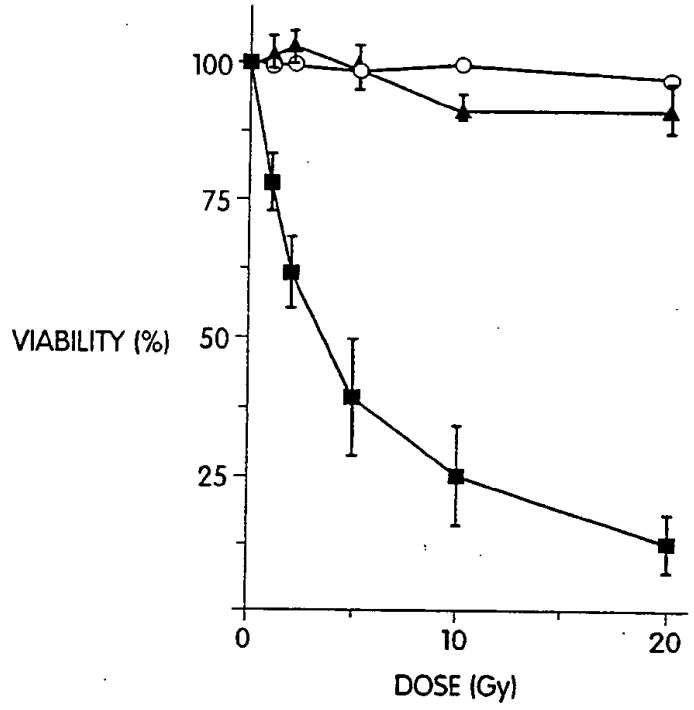

100
75
50
25
VIABILITY (%)
0
10
20
DOSE (Gy)

Fig. 1A

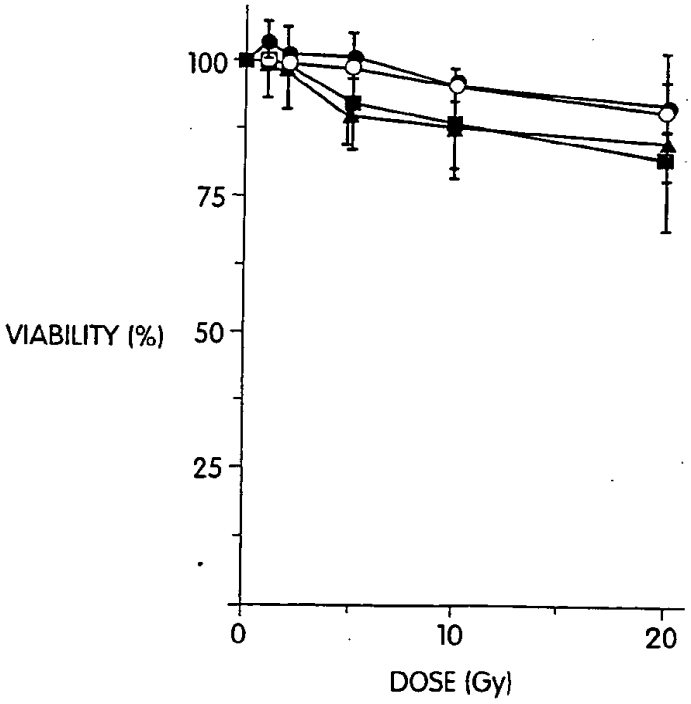

100
75
50
25
VIABILITY (%)
0
10
20
DOSE (Gy)

Fig. 1B

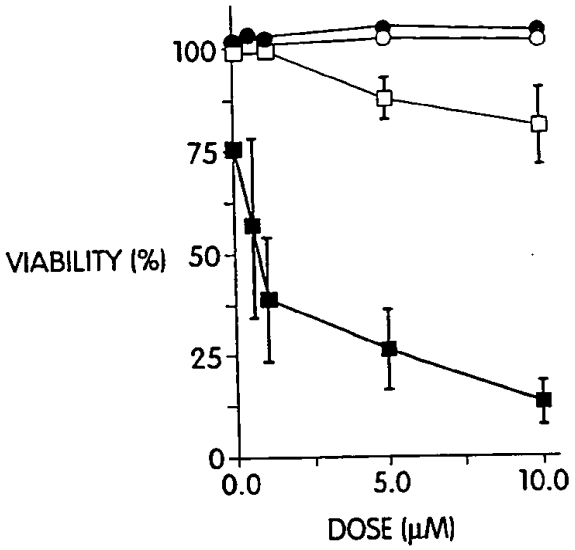



Fig. 2A

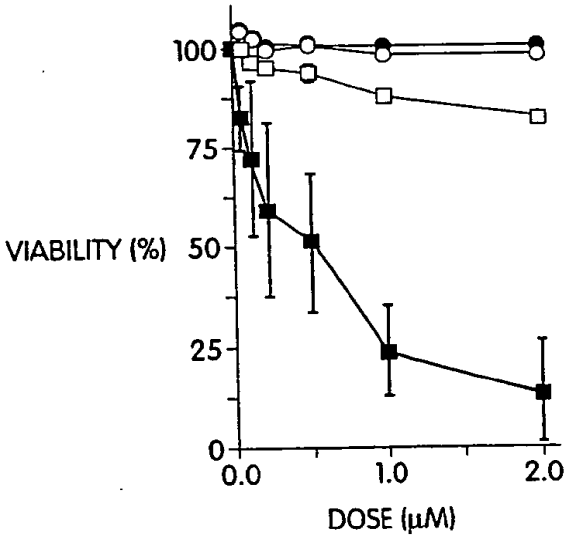



Fig. 2B

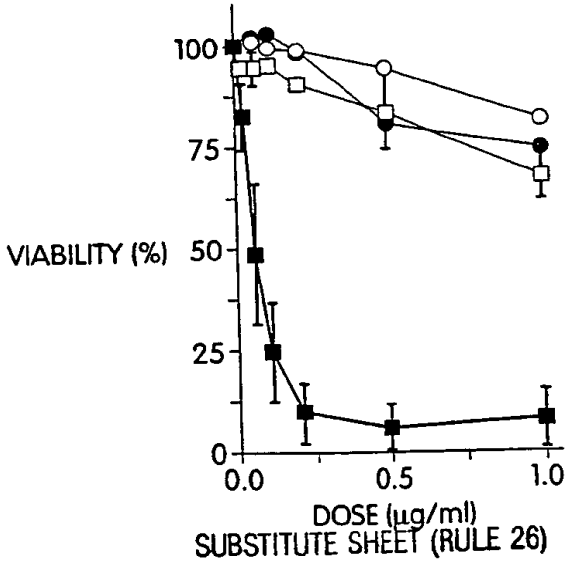



Fig. 2C

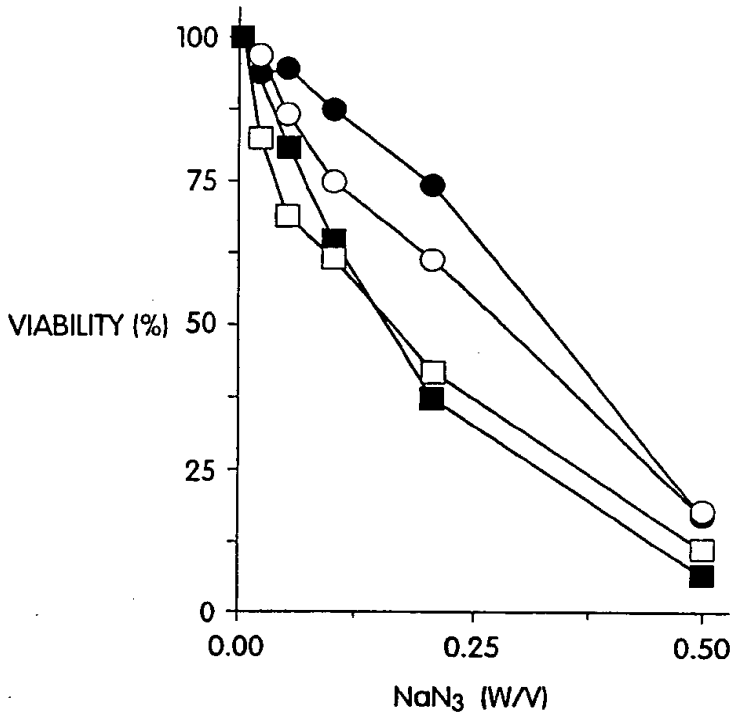

100
75
50
25
0
VIABILITY (%)
0.00
0.25
0.50
NaN3 (W/V)

Fig. 3

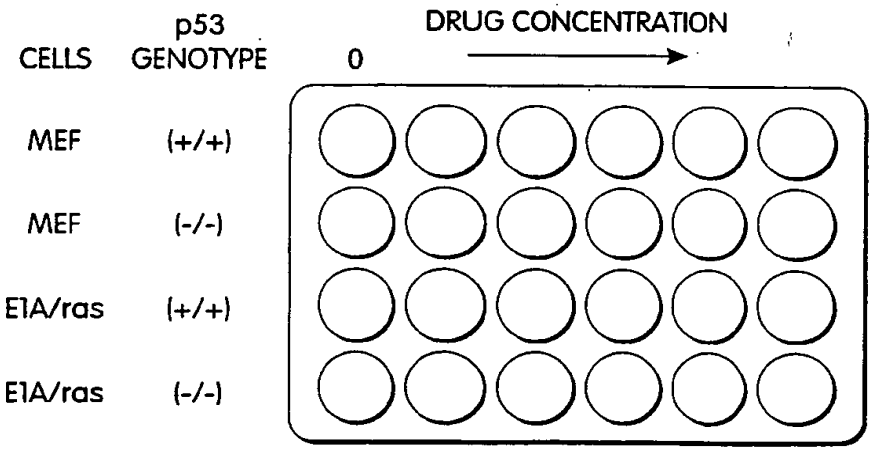

p53
CELLS
GENOTYPE
DRUG CONCENTRATION
0
MEF
(+/+)
MEF
(-/-)
E1A/ras
(+/+)
E1A/ras
(-/-)

Fig. 4

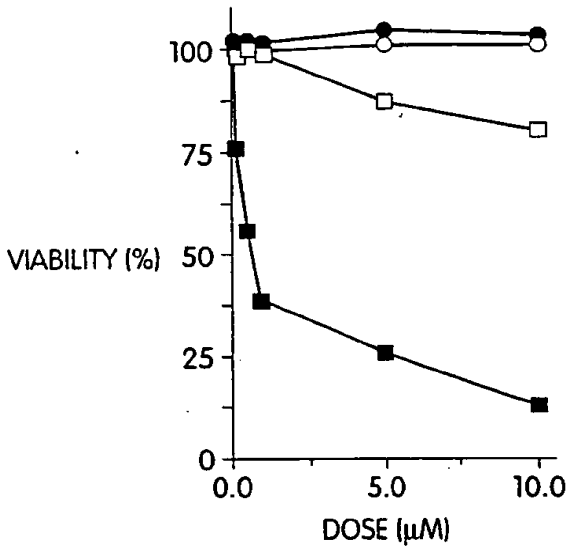



Fig. 5A

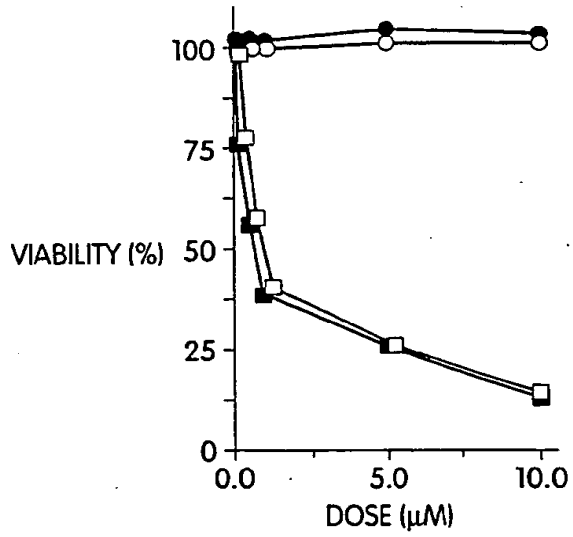



Fig. 5B

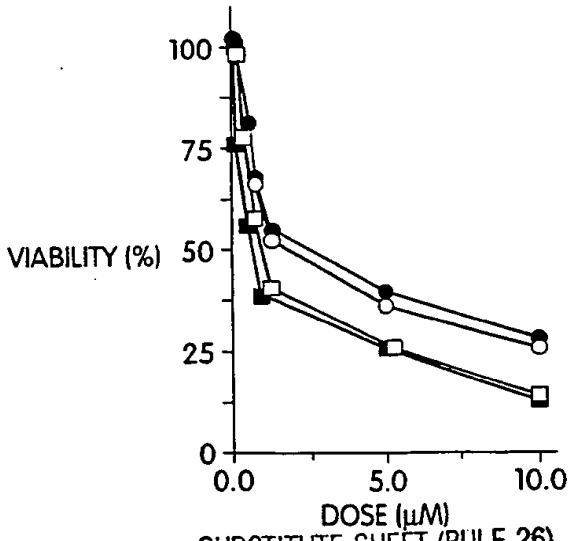



Fig. 5C

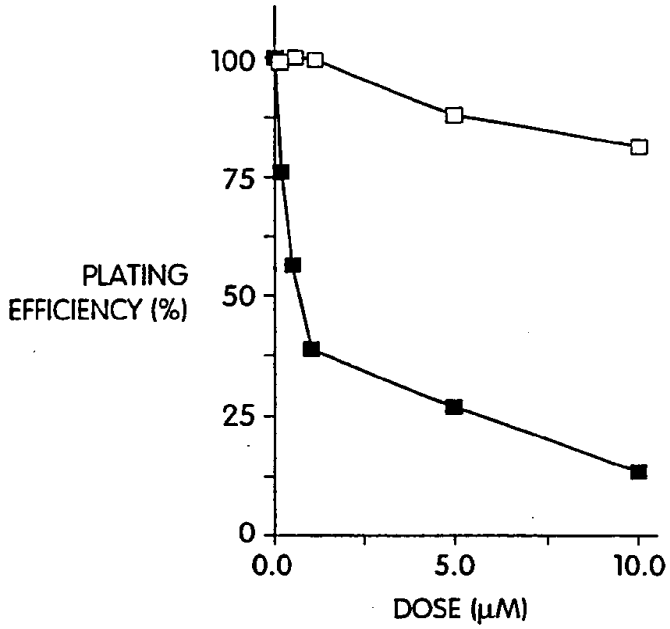

100
75
50
25
0
PLATING
EFFICIENCY (%)
0.0
5.0
10.0
DOSE (μM)

Fig. 6A

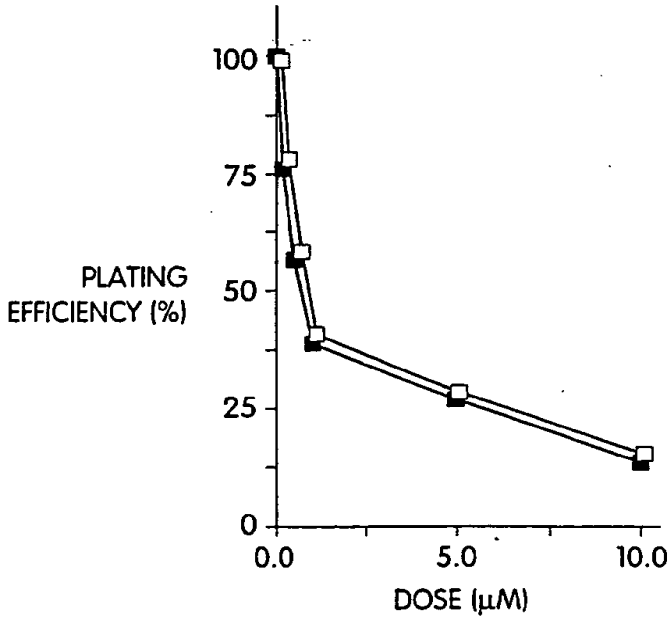

100
75
50
25
0
PLATING
EFFICIENCY (%)
0.0
5.0
10.0
DOSE (μM)

Fig. 6B

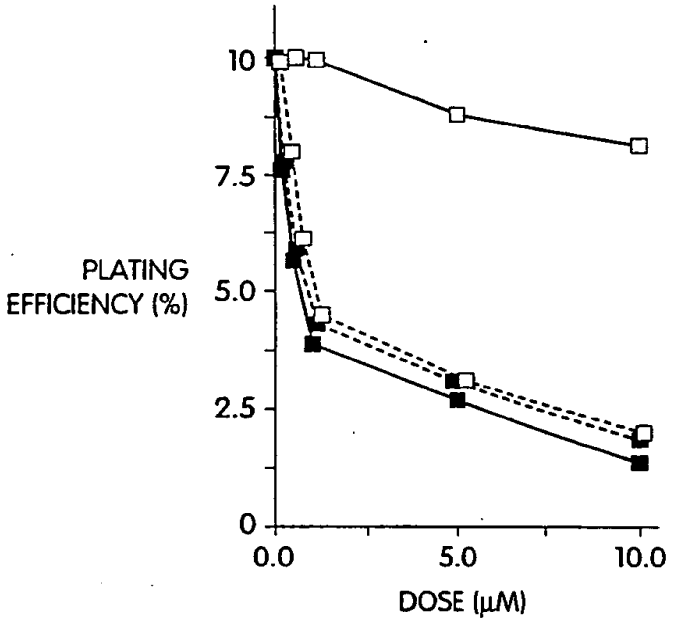



Fig. 7

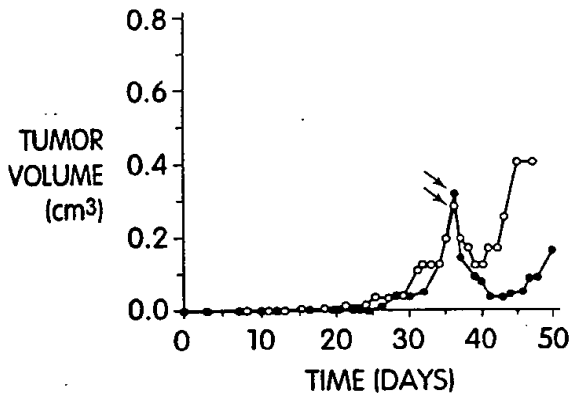



Fig. 8A

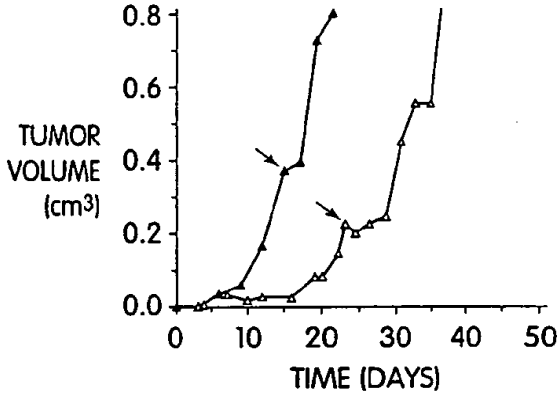



Fig. 8B

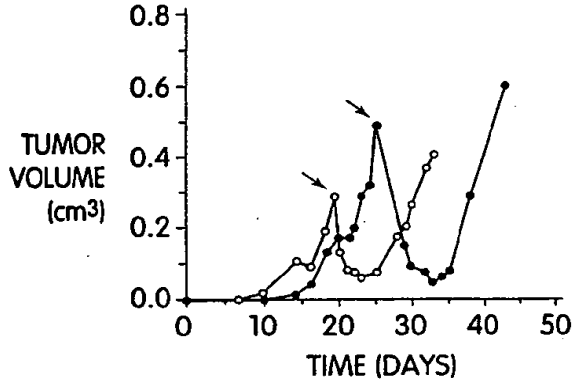



Fig. 8C

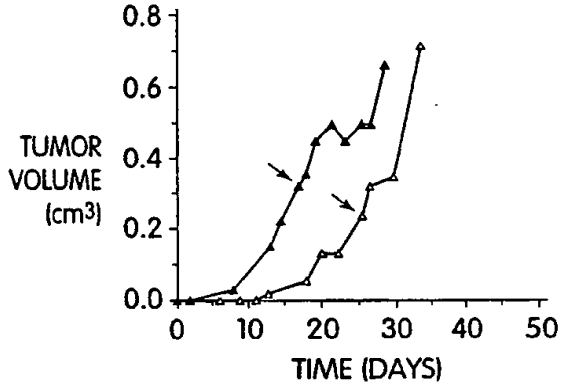



Fig. 8D

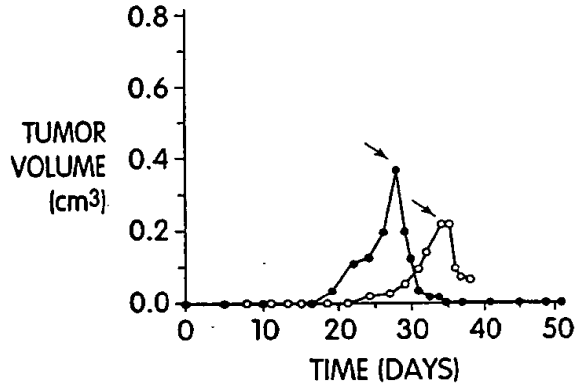



Fig. 8E

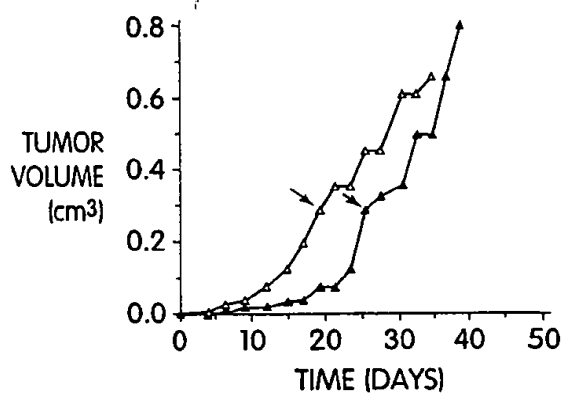



Fig. 8F

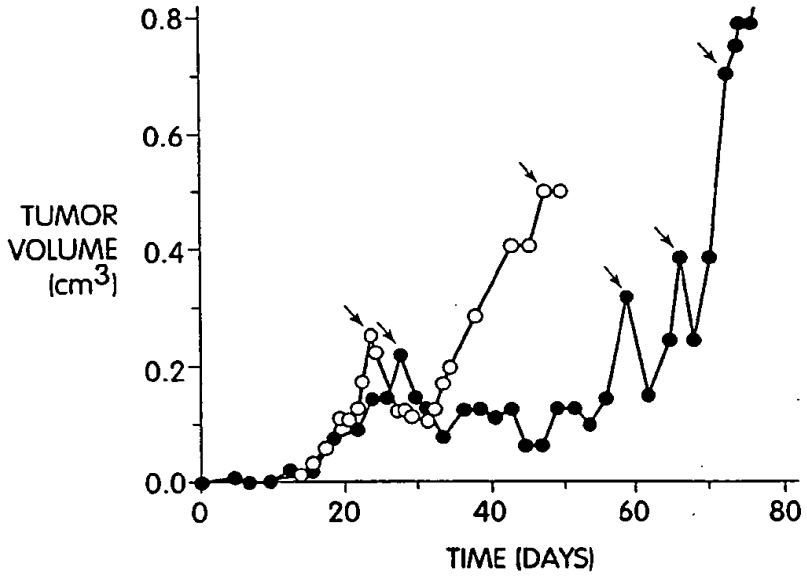

0.8
0.6
0.4
0.2
0.0
TUMOR VOLUME (cm3)
0
20
40
60
80
TIME (DAYS)

FIG. 9A

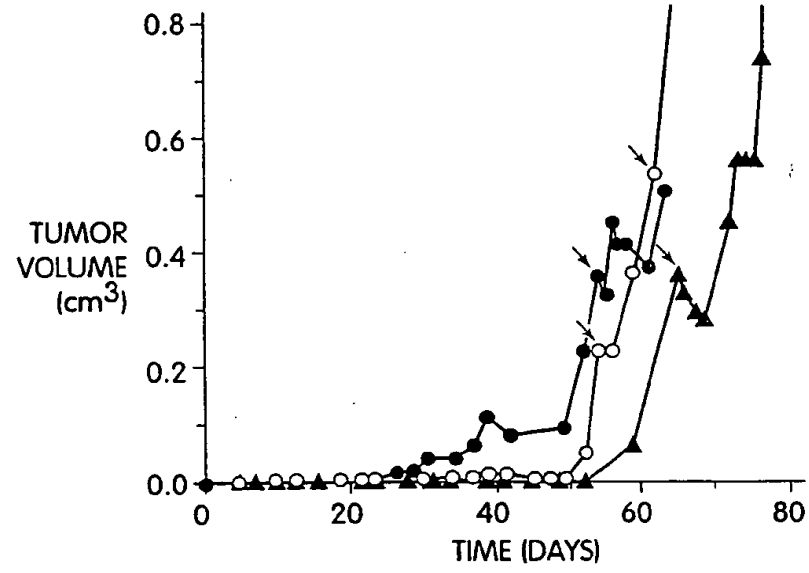

0.8
0.6
0.4
0.2
0.0
TUMOR VOLUME (cm3)
0
20
40
60
80
TIME (DAYS)

FIG. 9B

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09439

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A01N 43/38; A61K 31/40
US CL :514/410
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/410

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS file Medline

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | BLOOD, Volume 82(1), issued 01 July 1993, Sachs L. et al., "Control of Programmed Cell Death in Normal and Leukemia Cells: New Implications for Therapy", pages 15-21, see Medline Abstract No.: 93313190 only. | 1-50 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>17 NOVEMBER 1994 | Date of mailing of the international search report<br>12 DEC 1994 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>PAUL KULLOS jd<br>Telephone No. (703) 308-1235 |

Form PCT/ISA/210 (second sheet)(July 1992)*